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Biomarkers of salivary gland disease in Sjögren's syndrome

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Biomarkers of salivary gland disease in Sjögren's syndrome

**Thesis submitted for the degree of Doctor of
Philosophy**

By

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2017

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Abstract

Sjögren's syndrome (SS) is a systemic, chronic, autoimmune inflammatory disease that affects the exocrine glands. The absence of early diagnostic markers contributes to delays in its diagnosis. Furthermore, SS patients have an increased incidence of lymphoma and there is a need for biomarkers to identify its development. To enable a better understanding of disease activity and progression, the following aims were undertaken; 1. Investigate the potential use of the salivary gland assessment tests (whole flow rates (WFR), parotid flow rates (PFR), clinical oral dryness score (CODS) and ultrasound score (USS)) in discriminating SS and non- SS sicca patients as well as to differentiate between the different subgroups of SS. 2. Determine the diagnostic accuracy of USS. 3. Complete a longitudinal study on dry mouth patients at 5 and 10-year time-points. 4. Identify salivary markers of disease activity and progression in SS; MALT-L risk and MALT-L subgroups.

Methodology: Clinical parameters (WFR, PFR, CODS and USS) of 244 patients involved in the cross- sectional study were recorded for SS of different subgroups as well as for disease controls and ROC curves were constructed to determine an optimal cut- off USS. Five-year follow- up of 80 patients was done while only 16 were involved in the 10-year follow- up.

Salivary (parotid) proteomic analysis was done followed with initial candidate biomarker selection (S100A8/A9) and verification via immunoassay (ELISA) involving SS of different subgroups, disease and healthy controls (n=83). Salivary (parotid) cytokine array analysis was done followed by further screening of cytokines via multiplex bead- based assay on 76 samples and verification of the cytokines with significantly altered levels via a more sensitive multiplex bead- based performance assay on 82 samples.

Results: 1. All parameters (WFR, PFR, USS and CODS) of the overall SS group showed a significant difference when compared to the disease control group. This was attributed mainly to the advanced subgroups of SS (SS at risk and MALT-L groups) ($p < 0.0001$ for all parameters). 2. USS would be an ideal non-invasive test to differentiate and monitor SS

patients in general; an optimal cut- off of 4 yielded 81% sensitivity and 94% specificity and odds ratio of 70.5% with a negative predictive value of 66% while the positive predictive value was 97%. 3. The follow- up of patients over 5 and 10 years has proven that in patients with a longer disease duration SS is a slowly progressing disease where all the parameters remained relatively stable, although some individuals may display flare ups and remissions as in any other rheumatic diseases.

S100A8/A9 ELISA analysis of parotid saliva showed significant differences between the overall SS group and both disease and healthy controls ($p=0.001$ and 0.031 respectively). SS at risk of MALT-L and those with MALT-L also demonstrated increased levels when compared to healthy controls ($p=0.019$ and 0.014).

IL-1 α , -4, -6, and MCP-1 were significantly different in the overall SS group when compared to the disease control group but only IL-6 was increased when compared to the healthy control group. Further verification via more sensitive assays revealed that IL-6 and IL -4 continued to show significance compared to the disease control group and IL-6 compared to the healthy group.

Conclusion: the results of the above-mentioned studies suggest that several clinical parameters can aid in differentiating between SS and non- SS sicca especially the subgroups of SS (MALT-L risk and MALT-L sub groups) with attention to USS as a valuable diagnostic tool. Salivary biomarkers showed differences between SS and the other groups as well.

List of awards and presentations

- Best oral presentation at the Plenary session of Post graduate research day (PGR) at King's College London, March 2015.
- Poster presentation at the International Association of Dental Research/ Pan-European Regional (IADR/PER) Congress in Dubrovnik, Croatia, September 2014.
- Poster presentation at the British Society of Dental Research (BSODR) Annual Conference in City Hall, Cardiff, September 2015.
- Poster presentation at the 9th Saudi Student Conference (SSC) in Birmingham, February 2016.
- Poster presentation Biennial Congress of the European Association of Oral Medicine (EAOM) September 2016 (presented by Dr Pepe Shirlaw).
- Abstract "Parotid salivary biomarkers of Sjögren's syndrome" Oral Diseases 2016 volume 22, Issue Supplement S2, Pages 1-54.

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Abbreviations

ACR	American college of rheumatology
AECG	American-European Consensus Group
AID	Autoimmune disease
AIDS	Acquired Immunodeficiency Syndrome
ANA	Antinuclear antibody
Anti-M3R	Anti-muscarinic type 3 receptors
APRIL	A proliferation-inducing ligand
BAFF	B cell activating factor
BCA	Bicinchoninic Acid assay
BCA-1 (CXCL13)	B cell-attracting chemokine
CMV	Cytomegalovirus
CODS	Clinical Oral Dryness Score
DCs	Dendritic cells
DCT	Disease control
DEMS	Dry eyes and mouth syndrome
DIH	Drug induced hyposalivation
DLBCL	Diffuse Large B-cell lymphoma
EBV	Epsteine Barr virus
EC	Epithelial cells
ELISA	Enzyme linked immunosorbent assay
EPR	Electronic patient records
ESSPRI	EULAR Sjögren's syndrome patient reported index
EULAR	European League Against Rheumatism
FLS	Focal lymphocytic sialadenitis
FLT3L	Fms-like tyrosine kinase 3 ligand
FNA	Fine needle aspiration
GC	Germinal centres
GCF	Gingival crevicular fluid
HCV	Hepatitis C virus
HHV-6	Human herpes virus type 6
HHV-8	Human herpes virus type 8
HIV	Human immunodeficiency virus
HRP	Horse radish peroxidase
HTLV-1	Human T lymphotropic virus type I
IFN	Interferon
IP-10 (CXCL10)	IFN-gamma-inducible protein
KCS	Keratoconjunctivitis sicca
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LED	Light-emitting diodes
LSG	Labial Salivary Gland Biopsy
MALT-L	Mucosa associated lymphoid tissue lymphoma
mDC	Myeloid dendritic cells
MESA	Myoepithelial sialadenitis
MIP-3-β (CCL19)	Macrophage inflammatory protein-3-beta

MRI	Magnetic resonance imaging
MZLs	Marginal zone B- cell lymphoma
NHL	Non-Hodgkin lymphoma
NS	Not otherwise specific
OS	Overall survival rate
OSS	Ocular staining score
PBC	Primary biliary cirrhosis
pDC	Plasmacytoid dendritic cells
PFR	Stimulated parotid flow rate
RA	Rheumtoid arthritis
REAL	The Revised European- American Lymphoma
RF	Rheumatoid factor
ROC	Receiver operator characteristic
SDF-1 (CXCL12)	Stromal cell-derived factor- 1
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis
SGUS	Salivary gland ultrasonography
SICCA	Sjögren's International Collaborative Clinical Alliance
SIR	Standardised incidence ratio
SLC (CCL21)	Secondary lymphoid-tissue chemokine
SLE	Systemic lupus erythematosus
SNOX	Sialadenitis, nodal osteoarthritis & xerostomia
SPS	Stimulated parotid saliva
SS	Sögren's syndrome
Th cell	T helper cell
TIC	Total ion current
TLR	Toll like receptor
USS	Ultrasound score
UWMS	Unstimulated whole mouth saliva
VAS	Visual analogue scale
WFR	Unstimulated whole flow rate
WHO	World Health Organization

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1 Introduction

Sjögren's syndrome (SS) (also known as autoimmune exocrinopathy and autoimmune epitheliitis) is a systemic, chronic, inflammatory, autoimmune disease that affects the exocrine glands, particularly the salivary and the lacrimal glands. The pathogenesis of the disease remains not fully understood and to date no universally effective therapy is available. Clinically, dry mouth (stomatitis sicca or xerostomia), dry eyes (keratoconjunctivitis sicca (KCS) or xerophthalmia) and fatigue with /without inflammatory arthritis are among the most common manifestations. Extra glandular manifestations can occur in SS along with glandular manifestations which may affect the patients' quality of life. Occurrence of mucosa associated lymphoid tissue lymphoma (MALT-L) is one of the most serious manifestations of SS. Histologically SS, is characterised by focal lymphocytic infiltration of the salivary and lacrimal gland with concomitant destruction of the glandular tissue as shown in **figure 1-1**. The disease can occur by itself or with an accompanying autoimmune disease (AID), most commonly rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (Fox, 2005).

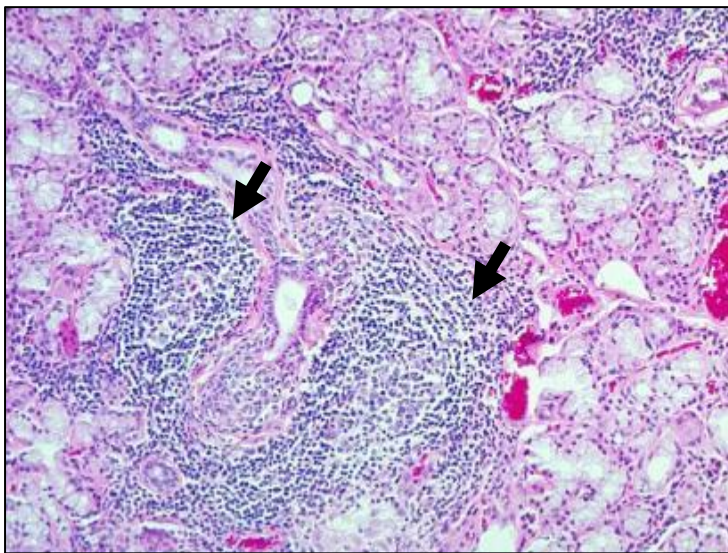


Figure 1-1 Histopathological lesion in minor salivary glands of a patient with SS. The hallmark of SS is periductal lymphocyte infiltration of the salivary glands
Focus Score= Number of lymphocytic aggregates (≥ 50 cells) per $4 \text{ mm}^2 \geq 1$ (Chisholm and Mason, 1968).

1.1 Background

1.1.1 Historical view of Sjögren's syndrome

The history of Sjögren's (Mikulicz, Gougerot's, Houwer, and Sicca amongst others) syndrome goes back to more than 100 years ago; a German physician named Leber, first described one of its components (Filamentary keratitis) in 1882. Between that year and 1927, several clinicians [Hadden in 1888, Fischer in 1889, Gougerot in 1925, Houwer in 1927 and others] reported similar cases with similar features. Mikulicz in 1892, reported a case of bilateral parotid and lacrimal enlargements with round cell infiltrations. The term Mikulicz's syndrome could cover many different entities thus it did not provide sufficient prognostic or therapeutic information. However, the term is still used intermittently to describe the histological appearance of focal lymphocytic infiltrates in salivary-gland biopsy samples. In 1933, a full description was made by a Swedish ophthalmologist named Henrik Sjögren in his doctoral thesis in which he presented clinical and pathological findings of 19 patients (reached 80 in 1951) complaining of dry mouth, dry eyes and arthritis, the term keratoconjunctivitis sicca was first introduced by him as well. Ten years later (1943), Hamilton translated it into English under the name of 'a new concept of keratoconjunctivitis sicca' (Bunim, 1961, Fox, 2005).

1.1.2 Epidemiology

Incidence and prevalence rates of SS vary widely around the world depending on the classification criteria used (Qin et al., 2015). Few global studies have been conducted, describing the epidemiology of SS and the regional difference are largely not known. The use of different sample sizes and criteria across these studies probably lead to the variation in the reported incidence and prevalence rates (being higher in earlier studies than in newer studies), which causes difficulty in interpretation and comparison of their results. In Europe, Bolstad and Skarstein, (2016) reported a prevalence of ~0.04 % from an oral perspective when the American European Consensus group (AECG) criteria were only used (Vitali et al., 2002). However, when different criteria were combined from different countries, it was found to be from 0.03 to 2.7 (95%CI 1- 4.5) (Patel and Shahane, 2014). In the United States, prevalence

of 0.02-0.1 was reported (Maciel et al., 2016). Another study reported a prevalence range of 0.2-3.0% which was generated from previous studies with a yearly incidence of 3.9 per 100.000 reported by other 2 studies . The female-to-male ratio of SS is 9:1 SS with an onset that typically occurs in the fourth to sixth decades of life (Reksten and Jonsson, 2014).

1.2 Clinical features

The clinical manifestations of SS may vary and the onset is often insidious, it can be non-specific during the early stages of the disease which might be overlooked or mistaken; accordingly, diagnosis may therefore be delayed for many years. The main clinical characteristic features of primary SS are the sicca symptoms which are progressive dryness of the eyes (KCS) and dryness of the mouth (xerostomia). Both of which are part of the exocrine glandular features (lacrima and salivary glands) (Kassan and Moutsopoulos, 2004).

1.2.1 Exocrine glandular manifestations:

Ocular manifestations: include dryness of the eye; sensation of gritty (sandy), itchy or sore eyes which can appear as normal or red eyes, discharge from the eyes, eye fatigue, inability to cry, eyelid adherent in morning and increased sensitivity to light, all of which affecting the person's ability to watch and read. Superficial corneal erosions may occur and may become as severe as filamentary keratitis, which is caused by the adherence of mucus filaments to the eroded (damaged) areas of the eyes. In addition, patients may develop conjunctivitis via Staphylococcus aureus infection and rarely manifest lacrimal gland enlargement (Kassan and Moutsopoulos, 2004).

Oral manifestations: xerostomia (dry mouth) is the main symptom and although this subjective complaint of xerostomia does not necessarily correlate with objective measures of hyposalivation, it is rather correlated with compositional change of saliva (Billings et al., 2016). Fox et al., (2008) reported xerostomia in 90% of their patients and if dry mouth is truly present it may lead to numerous complications summarized in **table 1-1**; extra- oral findings include cracked lip and angular cheilitis and intra- oral signs that range from subtle to obvious. Vissink

et al., (2003) reported a strong correlation between tear and saliva quality and secretion rate. It is well known that xerostomia has a huge effect on the person's quality of life in terms of its physical, psychological, and social aspects. Patients might not complain of dryness but of unpleasant taste and mouth soreness which might obscure the diagnosis for a period of time. Salivary gland enlargement might occur alongside as well; which might be associated with the increase risk of lymphoma development (Kassan and Moutsopoulos, 2004)(figure 1-2).

Other oral lesions of autoimmune aetiology (e.g. lichen planus and pemphigus vulgaris) might occur in 12% of SS patients. These disorders often cause ulceration and irritation of the oral tissues ranging from mild to severe (Likar-Manookin et al., 2013).

Additional exocrine glands: other glands are affected as well and appear as respiratory, vaginal and skin reduced glandular-secretions; nose, throat, tracheal dryness resulting in nasal crusting, epistaxis, recurrent sinusitis, dry cough and dyspnoea. Vaginal dryness might cause dyspareunia and pruritus whilst skin dryness may result in skin rash or burning and ulcerations (Kassan and Moutsopoulos, 2004).

Table 1-1 Clinical features of xerostomia

Oral consequences of xerostomia	
Dry sore mucosa (stringy saliva)	Gross accumulation of plaque
Difficult swallowing and speaking	Increased frequency of caries(cervical)
Reduce denture retention	Liability to Candidal infection
Burning fissured/lobulated tongue	Gingivitis
Dry cracked sore lips	Traumatic oral lesions
Ascending sialadenitis	Mucosal atrophy
Salivary gland enlargement	Halitosis

Modified from Kassan and Moutsopoulos,(2004) and Mathews et al., (2008).

1.2.2 Extra-glandular manifestations

Typical but rarely diagnostic, extra-glandular manifestations can occur due to the typical lymphocytic infiltration around the epithelium of target organs and because of the circulating autoantibodies. They develop slowly with variable outcome, and in some cases the on-going pathologic process may lead to severe organ damage and end-stage organ failure (Kassan and Moutsopoulos, 2004).

The prevalence of these manifestations varied greatly between the studies with a range of >10- 50% of SS patients. They include non -specific features such as fever, fatigue, malaise, lymphadenopathy and musculoskeletal involvement such as arthralgias, myalgias and myopathy. Some of the visceral manifestations include the following: pulmonary (bronchitis/bronchiolitis), renal (e.g. interstitial nephritis), gastroenterologic (e.g. oesophageal dysmotility), cardiovascular (e.g. pericarditis) and neurologic (e.g. peripheral sensory neuropathy and central nervous involvement; acute and chronic myelopathies) (Kassan and Moutsopoulos, 2004). Among them the most common symptoms include numbness and tingling of the extremities, Raynaud's phenomenon, malaise, lymphadenopathy and gastrointestinal disturbances also fatigue was reported amongst the most common complaints (Vissink et al., 2012).

Furthermore, cutaneous vasculitis is another skin manifestation that is considered as one of the most common extra-glandular features manifesting as palpable or non-palpable purpura. It is associated with the presence of hypergammaglobulinemia and related to a benign B-cell proliferation. On the other hand, cryoglobulinemic type of cutaneous vasculitis might occur, it is a systemic immune complex-mediated vasculitis with complement activation and can be used as a predictor of lymphoma; as it is considered as a prelymphomatous condition in SS (Quartuccio et al., 2015) (**figure 1-3**).

(A)



(B)



(C)



(D)



Figure 1-2 Extra- and intra oral manifestations.

(A) Angular cheilitis (B) Parotid gland swelling (C) lobulated tongue (D) Cervical caries.

(A)



(B)



Figure 1-3 Extra- glandular manifestation (cutaneous vasculitis)

1.2.3 Accompanying autoimmune disease

SS can be associated with organ specific autoimmune disease such as primary biliary cirrhosis, autoimmune thyroid or gastric disease. Furthermore, SS can develop secondary to another connective tissue autoimmune condition. In some patients, SS may precede the other disease by many years. It is sometimes difficult to determine whether a clinical manifestation is solely a consequence of SS or is due to other autoimmune disease. The combination does not modify the prognosis or outcome of the other autoimmune condition. About 60% of SS patients have the disease secondary to another autoimmune disorder such as RA, SLE or systemic sclerosis. On the other hand, almost 25% of patients with RA or SLE have histologic evidence of SS (Kassan and Moutsopoulos, 2004). Conversely, the distinction between primary and secondary forms of SS, which is usually based on an early detection of the disease, may be now out-dated. It has become known that some individuals with one autoimmune disease have increased susceptibility to develop another. Thus, it seems of little use and may cause confusion to distinguish one autoimmune disease as secondary to another. Consequently, the diagnosis of SS should be given to all who satisfy the classification criteria while also diagnosing any organ-specific or multiorgan autoimmune disease occurring with it, without distinguishing SS as primary or secondary (Shiboski et al., 2012). **Table 1-2** summarises those accompanying autoimmune diseases.

Table 1-2 Accompanying autoimmune diseases in SS

Additional autoimmune diseases found in SS	
Hypothyroidism	Myositis
Graves disease/thyrotoxicosis	Renal tubular acidosis
Scleroderma	Glomerulonephritis
Pulmonary fibrosis	Idiopathic thrombocytopenia
Chronic active hepatitis	Coeliac disease
Primary biliary cirrhosis	Pernicious anaemia
Systemic lupus erythematosus	Rheumatoid arthritis

Adapted from Lazarus and Isenberg (2005)

1.3 Diagnosis of Sjögren's syndrome

Sjögren's syndrome is one of the most clinically diverse autoimmune diseases. Owing to its heterogeneous presentation, clinical management of SS remains a great challenge. Therefore, there is a great need to diagnose it with the ability to assess the disease activity accurately (Patel and Shahane, 2014). Early diagnosis of SS will prevent or ensure a timely treatment of many complications of the disease (Kassan and Moutsopoulos, 2004). It requires different areas of speciality practice. Due to the complex nature of the disease involving multiple sites, there is no single gold standard test for establishing the diagnosis of SS (Vitali et al., 2002). At present, the diagnosis usually depends on the combination of clinical and laboratory findings based on an expert opinion which will be discussed in the following section. Difficulty in diagnosing SS is explained in **figure 1-4**. The main differential diagnosis and exclusion criteria are summarized in **tables 1-3 and 1-4**.

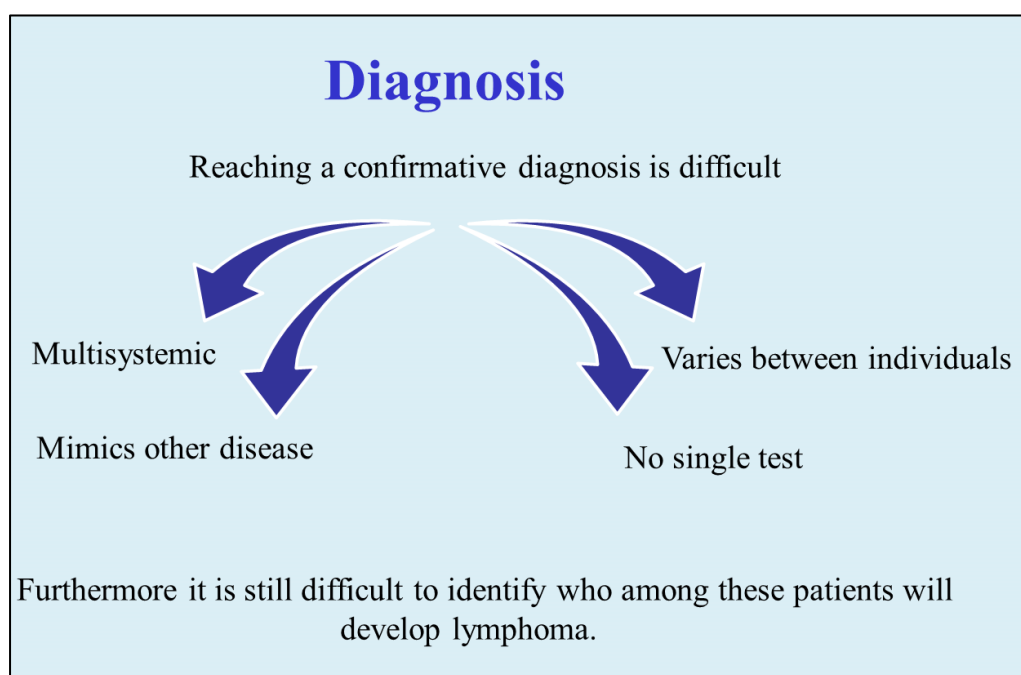


Figure 1-4 Difficulty in diagnosing Sjögren's syndrome

1.3.1 Diagnostic tests

Objective assessment of the ocular and oral features of SS is important to develop an accurate diagnosis:

1.3.1.1 Ocular tests

Ocular tests are performed to evaluate the tear volume (e.g. Schirmer's test), stability of tear film (e.g. tear break-up time) and the integrity of the ocular surface where selected semi-quantitative methods to assess the severity of KCS using vital dyes are used. Some of the modified tests lack validation and is not easily accessible also has low specificity, thus they are not accepted universally. However, Schirmer's test and Van Bijsterveld's ocular dye score (or any other dye) are part of the AECG criteria while ocular staining score (OSS) is part of the American college of rheumatology (ACR) criteria (Hernandez-Molina and Sanchez-Hernandez, 2013).

1.3.1.2 Oral tests

Oral tests are conducted to analyse the salivary gland function through sialometry and sialochemistry. The latter has an advantage over sialometry, which has the potential of showing these alterations in an early stage of the disease. It has been suggested as a diagnostic tool and the recent advances in mainly mass spectrometry systems have allowed several studies to identify several promising proteins and mRNA candidate biomarkers for SS (Al-Tarawneh et al., 2011). However, these markers need validation through further research (Hernandez-Molina and Sanchez-Hernandez, 2013).

According to Aframian et al., (2013) some of these sialochemistry studies showed different conflicting results due to different saliva collection methods, which warrants the need for a standardized collection protocol. Among these studies, it was found that in patients with SS, some proteins such as proline rich enzymes and carbonic anhydrase were down- regulated while the inflammatory proteins such as β -2 microglobulin and immunoglobulin (Ig) κ light chain were up- regulated some of which were validated pre-clinically (Hu et al., 2010).

Furthermore, it was reported that Anti- Ro60 and Ro52 autoantibodies can be detected in the saliva of SS patients with high specificity, suggesting a practical alternative to serum and compatible with point-of-care testing in patients with SS (Ching et al., 2011). Therefore, more evidence that supports using saliva biochemistry tests as a diagnostic tool is needed.

On the other hand, sialometry is easily performed and provides an objective assessment of the flow rate. Different sialometric tests were used; wafer test, Saxon test, oral Schirmer test, candy weight loss test, the palatal and specific glandular saliva flow, the capsaicin-stimulated salivary flow and the more traditional whole saliva flow with or without stimulus. The unstimulated whole flow rate test (WFR) is the only sialometric test that was included in the AECG criteria while none has been included in the ACR criteria, which found that WFR was unreliable diagnostic measure (Hernandez-Molina and Sanchez-Hernandez, 2013). It should be noted that glandular saliva can be more informative regarding disease progression and diagnosis (Pijpe et al., 2007, Vissink et al., 2012).

The Challacombe scale was developed from research conducted at King's College London Dental Institute, the Clinical Oral Dryness Score (CODS) uses a simple numeric system which enables the clinician to quantify the severity of the xerostomia and to decide if the condition needs treatment or not. This scale has been distributed to all general dental practitioners (Challacombe et al., 2015) (**Appendix 1**). Noting that earlier, Navazesh et al., (1992) has identified a set of 4 clinical parameters defining hyposalivation.

1.3.1.3 Oral and ocular symptoms scores

Oral and ocular scores are subjective measurements that can be determined using questionnaires and indices; they can be either non-specific (e.g. Ocular surface disease index) or disease- specific; (e.g. Sicca symptoms inventory). The subjective assessment of the AECG criteria included two sets of questions regarding ocular and oral symptoms and the EULAR Sjögren's syndrome patient reported index (ESSPRI) was used (Hernandez-Molina and Sanchez-Hernandez, 2013).

1.3.1.4 Histopathology examination

Histopathological examination of labial glands removed during a lower lip biopsy is a widely accepted diagnostic method where a focal lymphocytic sialadenitis (FLS) with a focus score of 1 or more is considered diagnostic for SS. Furthermore, the morphology contributes to the prediction of non-Hodgkin's lymphoma (NHL) development if there are lymphoid germinal centres present in the glands. However, it is an area of controversy, some studies have suggested excluding biopsies if patients were fulfilling enough criteria; clinical sicca symptoms/signs and positive serology. While others reported that biopsies had the highest predictive value which depends on the criteria being used (Vissink et al., 2012).

Shoboski et al., (2012) demonstrated that biopsy provides information about the extent and nature of the disease process. It has been criticized for being invasive and difficult to apply in all settings. However, being applied on nearly 1400 participants as part of their investigations would suggest otherwise. It is a minimally invasive 15-minute procedure, when it is skilfully and conservatively performed. Moreover, (Kapsogeorgou et al., 2013) found no change in repeated biopsies obtained over median period of 55 months indicating development of lymphoma in some patients with severe serological parameters. In addition, Carubbi et al., (2014) have identified an association between different histopathological patterns obtained via labial gland biopsies and different cytokine expression and clinical subsets.

It is worth noting that limitations of this procedure include difficulty in asking the patients to repeat the biopsy frequently for an assessment of the disease progression, possible permanent dysaesthesia of the lip, negative focus scores in patients fulfilling the diagnostic criteria for SS, variability of the histological interpretation and being inconclusive on occasions (Colella et al., 2010). Moreover, the reliability of such procedure was recently tested by Costa et al., (2015) demonstrated that the focus scores might be overestimated despite having a good reliability due to differences in assessment methods, and even with their importance for SS prognosis, germinal centres are often inadequately assessed.

To overcome these limitations parotid gland biopsy was suggested as an alternative way to diagnose SS, predict and confirm lymphoma development with several advantages; applicability of repeated biopsies of the same parotid gland, the results can be related with other diagnostic results derived from the same gland (e.g. sialometry, sialochemistry and sialography) and less sensory loss of skin (Vissink et al., 2012). Furthermore, measuring the effect of therapeutic intervention can be accomplished using sequential parotid biopsies. Pijpe et al., (2009) have demonstrated a histopathologic proof of glandular inflammation reduction. The importance of standardisation of laboratory techniques such as the cutting procedure via multiple cutting levels and an area of at least 4 mm², noting the presence of non-specific sialadenitis, ectopic germinal centres and fibrosis have been reported (Fisher et al., 2015).

1.3.1.5 Serological findings

Serological findings are used to diagnose SS and to assess its activity and progression as well as for prediction of lymphoma development, recognition of extra- glandular manifestation and evaluation of effectiveness of treatment. Hypergammaglobulinemia, several autoantibodies against non- organ specific antigen [rheumatoid factor (RF), antinuclear antibodies (ANA), extractable nuclear antigen (ENA) as SSA/Ro and SSB/La, rheumatoid arthritis-associated nuclear antigen] are common findings (Aframian et al., 2013).

Anti-SSA / Ro (52 and 60 kDa) and anti-SSB / La (48 kDa) were the most characteristic autoantibodies of SS (30–60%) and is accepted as a criterion of the AECG criteria (Vitali et al., 2002, Aframian et al., 2013). While RF and ANA levels were suggested by the ACR as a criterion that can substitute for ENA (Shiboski et al., 2012). In a most recent review by Ferro et al., (2016) it was demonstrated that others were reconsidering the addition of positive anti-SSB/La as SS diagnostic criterion where only 2 % of patients in a global cohort has positive anti-SSB/La and negative Anti-SSA/Ro. In addition, a number of other autoantibodies has been suggested as potential markers for SS diagnosis and severity, but they are more related to a specific extra-glandular manifestation of the disease (e.g. increased levels of carbonic anhydrase II Abs in patients with SS and distal renal tubular acidosis). Among them, two were

likely to have potential diagnostic value according to a review done by Aframian et al., (2013); Anti-muscarinic type 3 receptors (anti-M3R) Abs and α - fodrin Abs. Some reported a correlation between these Abs and disease severity and progression while others did not. They suggested screening for anti α - fodrin Abs with the routinely used anti-SSA/Ro and anti-SSB/La might be useful, particularly in those patients who are negative for anti-SSA/Ro and anti-SSB/La. Further research is needed to determine their diagnostic value (Aframian et al., 2013). Moreover, increased levels of IgG hypergammaglobulinemia, decreased complement levels C3 and cryoglobulinemia were amongst the predictive factors of lymphoma development and those aforementioned factors as well as anaemia, lymphopenia, thrombocytopenia and presence of monoclonal proteins were associated with extra-glandular manifestation such as splenomegaly, adenopathy and purpura. Regarding treatment effect, it was reported that RF levels were decreased following intervention as well as for B circulating cells. However, T regulatory cells have shown controversial results (Vissink et al., 2012).

1.3.1.6 Radiological tests

Imaging of the salivary glands includes conventionally X-ray sialography, scintigraphy, salivary gland ultrasonography (SGUS), computed tomography and magnetic resonance imaging (MRI). The first 2 tests are part of the AECG criteria (Vitali et al., 2002). When using an imaging test, a person should consider the cost, the invasiveness, the ionising radiation dose, the clinical usefulness, the applicability and more importantly the diagnostic accuracy. Sialography for diagnosing SS significantly depends on the skills of the observer, indicating that it is not generally applicable as a diagnostic tool in SS and requires specific expertise with a fair (inter-observer) agreement between trained and expert observers (Kalk et al., 2002b). Moreover, other conditions might mimic SS showing similar glandular appearance even in normal subjects (Kalk et al., 1999). Also it can be considered as an invasive technique; with ionizing radiation exposure, pain and swelling following the procedure, but a study by Kalk et al., (2001b) has shown that it has good patient acceptance, low morbidity and that it was not as invasive as it was often thought. Also, given its high sensitivity and specificity in diagnosing

SS as well as its useful monitoring potential, sialography remains the best performing imaging tool, more accurate than US and MRI in SS diagnosis. On the other hand, scintigraphy was poor compared to sialography, MRI and US with no standardised quantitative indices despite being as part of the AECG criteria (Vissink et al., 2012). SGUS or ultrasound score (USS) may offer a promising alternative as a valuable tool for detecting salivary gland abnormalities in SS; it is a feasible, easily accessible, inexpensive and non-irradiating imaging tool. Again, it requires an experienced clinician to interpret the images of SGUS thus it might not be easily applicable. It has been suggested to improve the diagnostic value of American-European Consensus Group (AECG) and American college of rheumatology (ACR) criteria. And it has been suggested as a first line of diagnosis by Salaffi et al.,(2008), showing a good correlation with sialography by Shimizu et al., (2006) and replacing scintigraphy (Milic et al., 2012). However, its reliability is still not clear yet and one of its limitations is difficulty of identifying the early stages of SS; inability to penetrate deep tissue as the sialography does. In order to enhance both the diagnostic and the monitoring potential of SGUS, it will require standardization and validation by comparing the SGUS scores of parotid and submandibular gland tissue with their flow rates in future studies (Vissink et al., 2012, Delli et al., 2015, Jousse-Joulin et al., 2016). A simplified scoring system (**Appendix 2**) was modified at King's College London Dental Institute radiology department (Brown, 2010). It was used in the first study (chapter 3), based on a system proposed by Hočevár et al., (2005).

Furthermore, other aspects of SS have been assessed, for example: -

- **Fatigue** is measured via various scales and indices among them the Visual analogue scale (VAS) is one of the most frequently used tools.
- **Quality of life** is evaluated via generic and some organ- specific measures.
- **Disease status** incorporates measurements of the activity of the disease (e.g. SS Disease Activity Index) and measurements of organ damage (e.g. SS Disease Damage Index) (Hernandez-Molina and Sanchez-Hernandez, 2013).

1.3.2 Classification criteria

Classification criteria have been developed for trial purposes mainly in clinical studies to guarantee the standardization of diagnosis, and to enable the analysis and comparisons of results of patients between institutions. These classification criteria aim for 100% sensitivity and specificity. However, classification criteria are often high in specificity but low in sensitivity. Classification criteria are being used for diagnosing SS. The criteria may not be reliable in the early stages of the disease where the characteristic features have not yet manifested. Thus, a certain proportion of patients may be misclassified. The heterogeneity of signs and symptoms has led to the development of multiple classification criteria.

Over the years, several sets of criteria have been proposed worldwide. 11 classification criteria have been reported since 1965; none have been accepted or validated by the ACR or the European League Against Rheumatism (EULAR). The most accepted criteria, which have been used and referred to, is the AECG criteria. Diagnosis is based on the concurrent presence of various signs and symptoms of the disease as established by 6 diagnostic standards: oral symptoms, ocular symptoms, evidence of oral signs, evidence of ocular dryness, evidence of salivary gland involvement, positive Anti-Ro/La autoantibodies and a positive gland biopsy (**Appendix 3**) (Aframian et al., 2013). The AECG criteria yielded a sensitivity of 96.1% and a specificity of 94.2% in primary SS diagnosis and in secondary SS had a sensitivity and specificity of 97.2% and 90.2% respectively. The criteria became broadly used and referred to, ever since (Vitali et al., 2002).

The ACR and EULAR criticized it for including subjective measures which can exclude asymptomatic patients, physiologic measures that lack specificity, and for alternating objective tests that differ in sensitivity and specificity. To address these issues and to develop a new classification, the on-going Sjögren's International Collaborative Clinical Alliance (SICCA) was established. Their classification was provisionally approved by the ACR.

According to the SICCA (ACR) criteria proposed in 2012, the diagnosis of SS requires at least 2 of the following 3 findings:

- Positive serum anti-SSA and/or anti-SSB antibodies or positive rheumatoid factor (RF) and antinuclear antibody (ANA) titre of at least 1:320.
- Ocular staining score (OSS) of at least 3.
- Presence of focal lymphocytic sialadenitis with a focus score of at least 1 focus/4 mm² in labial salivary gland biopsy samples (Shiboski et al., 2012).

The level of agreement between the preliminary ACR criteria and the AECG criteria was high when all objective tests were included to define the AECG criteria (88%). However, it was low when subjective tests were allowed to replace the objective tests (Shiboski et al., 2012).

According to Bowman and Fox, (2014), the high level of statistical agreement was due to the fact that recruitments to SICCA study included patients from support groups who possibly originally diagnosed by the AECG criteria. Researchers emphasized the need for establishing new classification criteria to support etiologic, genetic and therapeutic research trials. These criteria should be easily performed, be clear, have higher sensitivity/specificity and consider the potentially serious adverse effects and comorbidities of the new immunomodulating agents. They should rely on well- established conventional objective tests that are strongly associated with the different features of the disease and to be able to alternate between those tests only if they were comparable diagnostically. In addition, it was suggested that it was preferable for the new classification criteria to be acknowledged by the (ACR) and (EULAR). This was indeed recently developed and will be discussed later.

Vitali et al., (2013) reported some critical points about these criteria; firstly, that these new ACR criteria were not validated for secondary SS. Nevertheless, the new criteria were proposed as a valid tool to classify secondary SS according the SICCA study. Secondly, patients who fulfilled the AECG criteria might be misclassified due to the exclusion of subjective symptoms. Furthermore, the alternative use of ANA and RF instead of anti Ro and

anti La, which can be seen in another autoimmune disease and this warrants further studies. On the other hand, Vitali et al., (2002) suggested that the new ACR criteria which involve only 3 might be favoured over the more complex 6 domains (including 13 items) in AECG criteria. However, they explained that the AECG criteria are readily accessible and easy to use. The main criticism as mentioned before is alternating tests, which are not diagnostically equivalent.

It has been suggested that there should be a blend of the ACR and the AECG criteria to include both sets with different classifications options which then can be accepted by the ACR and EULAR (Bowman and Fox, 2014). Furthermore, several amendments to AECG criteria have been suggested; such as adding salivary gland ultrasonography (SGUS) to the AECG criteria (Cornec et al., 2013). Even though SGUS is a promising addition to AECG criteria not only for classification but also for diagnosis and monitoring the disease activity and progression nonetheless its role in the ACR classification criteria requires more research and should be tested in further studies as mentioned earlier (Bootsma et al., 2013).

At the ACR 2015 annual conference, C. Shiboski presented a new classification criteria which might replace the provisional ACR and AECG criteria and getting close to get accepted by the EULAR. This new criteria demonstrated great concordance with the AECG criteria (Ferro et al., 2016). The final classification criteria are based on the weighted sum of five items and a cut- off of 4 points would be considered SS:

- Positive serum anti-SSA antibodies (3 points)
- Ocular staining score (OSS) ≥ 5 or Van Bijsterveld score of ≥ 4 (1 point)
- Schirmer's test ≤ 5 in 5 min (1 point)
- Salivary flow rate ≤ 0.1 ml/min (1 point)
- Presence of focal lymphocytic sialadenitis with a focus score of at least 1 focus/4 mm² in labial salivary gland biopsy samples (3 points) (Shiboski et al., 2017).

1.3.3 Disease that may underlie dry mouth, dry eyes or parotid gland enlargement

Table 1-3 Possible conditions leading to:

Dry mouth	Dry Eyes	Bilateral Parotid Gland Enlargement
Viral infections	Inflammation: (chronic blepharitis or conjunctivitis, pemphigoid, or Stevens-Johnson syndrome)	Viral infections: (Mumps, Influenza, HCV, HIV)
Drugs*		
Radiation		
Amyloidosis	Amyloidosis	Amyloidosis
Sarcoidosis	Sarcoidosis	Sarcoidosis
Trauma	Blink abnormality	Metabolic diseases: Diabetes mellitus Chronic pancreatitis Hepatic cirrhosis Hyperlipoproteinemias
Psychogenic	Eyelid scarring	
Diabetes mellitus	Toxicity (burns or drugs)	
SS	Trauma to the eyes	Endocrine disorders: Acromegaly Gonadal hypofunction
	Tumors	
	Neurologic conditions	SS
	Corneal anaesthesia	
	Hypovitaminosis A	
	SS	

*antihypertensive, parasympatholytic, and psychotherapeutic agents.
Modified from Kassan and Moutsopoulos, (2004).

Table 1-4 Exclusion criteria according to the AECG and ACR criteria

Exclusion criteria	
(AECG)	(ACR)
Sarcoidosis	Sarcoidosis
Graft versus host disease	Graft versus host disease
Hepatitis C infection (HCV)	Hepatitis C infection (HCV)
Acquired Immunodeficiency Syndrome (AIDS)	(AIDS)
Head and neck radiation	Head and neck radiation
Pre-existing lymphoma	Amyloidosis
Anticholinergic drugs (since a time shorter than 4-fold the half-life of drug)	IgG4-related disease

AECG; American-European Consensus Group (Vitali et al., 2002)

ACR; American College Rheumatology (Shiboski et al., 2012)

1.3.4 Sialadenitis, nodal osteoarthritis and xerostomia (SNOX) as different disease entity

In a study by Kassimos et al., (1997), patients from an Oral medicine clinic setting were diagnosed provisionally with SS but proven not to satisfy the criteria upon examination and following their investigations (serology, histopathology and flows). The distinguishing features were; diffuse non-specific sialadenitis in their biopsies, seronegative anti-SS-A, anti-SS-B and Rheumatoid factor results with the presence of nodal osteoarthritis (NOA). As far as for the flow rate was concerned, whole mouth flow rates were reduced compared to normal range similarly to other SS patients (who satisfied the criteria). However, parotid flow rates were only reduced in some of the SNOX patients. SNOX patients were not on any known xerogenic drugs and fewer showed any ocular dysfunction (proven by Schirmer's test only).

The other part of the study established SNOX as a disease entity by studying age and sex-matched controls within a Rheumatology outpatient setting showing a statistically significant increase in the number of patients with NOA and xerostomia. They also confirmed the presence of fewer cases of ocular dysfunction. NOA is characterised by polyarticular osteoarthritis which is mainly inflammatory. Twenty-five per cent of NOA patients had SNOX features suggesting it as a distinct disease entity. Erosive osteoarthritis was linked to SS. However, it is clearly that SS patients can also have osteoarthritis but SNOX can be distinguished by the other features mentioned earlier. Another study has reported a positive correlation between the reduced flow rate and reduced mucosal wetness and with increased CODS seen in different dry mouth patients (including SNOX) (Osailan et al., 2011). A similar group of patients was described by another study and they termed these patients as having dry eyes and mouth syndrome (DEMS) (Price and Venables, 2002). SNOX was chosen as a disease control group for the following chapters in order to verify its presence among dry mouth patients while comparing it to SS patients.

1.4 Pathogenesis

Investigators have considered SS as an immune mediated salivary gland dysfunction prior to glandular destruction. Tissue loss due to apoptosis, fibrosis and atrophy of the salivary glands would signify the classical model of gland hypofunction in SS. A non-apoptotic model was introduced in which atrophy is a result of glandular hypofunction not a cause of it and this explains the presence of normal acinar cells in SS patients with glandular hypofunction (Dawson et al., 2006). It is characterized by both T- cell lymphocytic infiltration of the exocrine glands and B-cell lymphocyte hyperactivity. However, the mechanism by which this action takes place is still unknown. Furthermore whether this hyperactivity is a primary cause or secondary effect in SS is not known either (Jonsson et al., 2011).

Generally, the development of SS may be divided into three stages:

- Activation of autoimmunity by different aetiological factors.
- Initiation of the immune system (innate and acquired) and an increase in the chronic autoimmune response.
- Tissue damage (Pers et al., 2012).

1.4.1 Aetiological factors

Although the pathogenesis of SS is still unclear, the following factors have been suggested:

Environmental factors: it has been postulated that viral infections such as cytomegalovirus (CMV), Epstein Barr virus (EBV), retroviral elements, human herpes virus type 6 (HHV6), human T lymphotropic virus type 1 (HTLV-1), human herpes virus type 8 (HHV-8) and recently, coxsackie viral sequences, play a role as triggering factors, still controversial results were found between studies (Tzioufas et al., 2012).

It is worth noting that infections with certain viruses as hepatitis C virus (HCV) and human immunodeficiency virus (HIV) might lead to inflammation of the salivary glands mimicking that of SS without the production of autoantibodies (anti-SSA/SSB).

The genetic predisposition: it varies among ethnic groups in SS and studies have found higher incidence of SS in family members and a higher prevalence of serological autoimmune abnormalities than controls. As with other autoimmune diseases a strong association to specific major histocompatibility complex (MHC) alleles has been demonstrated as well as the presence of genetic polymorphism (Tzioufas et al., 2012). Studies on the role of epigenetics have been extensively growing; it was found that in SS patients a global DNA methylation is reduced in the epithelial cells of salivary gland also microRNA expression profile is altered in SS patients compared to controls (Cornec et al., 2014).

Hormonal factors: the strong female predominance observed in SS, suggests an influence of sex hormones but their role in the pathogenesis of SS remains unknown, however it seems that estrogenic deficiency predisposes to SS, thus might explain the development of the disease during the peri-menopausal period of life of females (Tzioufas et al., 2012).

1.4.2 Immunopathology

1.4.2.1 Cellular pathways

It is assumed that infection as a trigger (most commonly viral) and other factors (mentioned above) caused the disorganisation of epithelial cells (ECs). Once triggered damaged or/and dead, EC will have a significant role in the pathophysiology as a regulators of the inflammatory process (aberrant homing of auto reactive B-cells and T- cells), it was speculated to be involved in the initial steps of the pathogenesis hence the name autoimmune epitheliitis was suggested as an etiological name of the disease (Tzioufas et al., 2012).

These triggered EC activate the HLA-independent innate immune system whereby they release triggering auto-antigens (e.g. SS-A protein and SS-B) to Toll-like receptors (TLR), which recognize pathogen-associated patterns (present in several microorganisms and apoptotic products) and they act as antigen presenting cells together with the adjacent T- cells and begin producing pro-inflammatory cytokines that up-regulate chemokines, and adhesion molecules production in the gland. These changes will direct lymphoid migration into the gland where the HLA-dependent acquired immunity takes place which involves lymphoid cell

recruitment, homing, activation, differentiation and proliferation, as well as the expansion and organization of lymphoid infiltrate. (The following graph explains it in full detail)

After migration of the lymphocytes and other cells such as dendritic cells (DCs) into the gland in response to chemokines and adhesion to specific adhesion molecules, they interact with each other and with the ECs. It is noteworthy that DCs have gained more interest in recent years, having a potential role in the pathogenesis. Immune complexes will be formed and will bind to the plasmacytoid (p)DCs through TLR, activating it and this will lead to production of Interferon (IFN)- α which is type-I IFN cytokine . This powerful modulator will further activate the epithelial cells and other immune cells (e.g. further homing and activation of lymphocytes). Studies have found an increased expression of IFN-regulated genes in SS patients (glands and bloods) compared to controls (Pers et al., 2012). This is so called the interferon signature that links both innate and acquired immunities. This was confirmed later by the presence of DCs in salivary glands as part of the inflammatory foci (Jonsson et al., 2011).

Then B lymphocyte migration, interaction with T cells and activation in the gland occurs under the influence of Th cells and cytokine B-cell activating factor (BAFF) that is produced in abundance by myeloid (m)DC which is modulated by the IFN- α thus causing aberrant B-cell maturation leading to the emergence of self-reactive B-cells serving as an antigen-presenting cells to T cells, and locally produce autoantibodies and rheumatoid factors. The on-going antigen stimulation can promote clonal expansion of B cells in the glands and blood, which might become malignant later. The presence of Ro/SS-A antibodies is correlated with longer periods of SS, greater damage of the glands and extra-glandular manifestation as mentioned previously (Maslinska et al., 2015). Some of the other signs and indicators of B-cell hyperactivity are presence of germinal centre-like structures in the glands, elevated glandular levels of B- cell associated cytokines (IL-6/ IL-21, BAFF/ a proliferation-inducing ligand (APRIL)) and chemokines (stromal cell- derived factor- 1 (SDF-1 or CXCL12), and B cell-attracting chemokine 1 (BCA-1 or CXCL13) as well as in saliva, tears and serum. Elevated

levels of soluble (s)CD27 in tissue while reduced number of memory B-cells in peripheral blood was reported amongst the indicators of B-cell hyperactivity (Kroese et al., 2014).

Analysis of infiltrates of salivary glands revealed majority of T cells surrounding the ductal epithelial cells with 80% CD4 cells and 10% CD8 (cytotoxic) while the remaining are B cells, DCs, macrophages and plasma cells. Their presence varies according to the severity of the disease. In mild lesions, the T cells are the majority while B cells are in advanced lesions. As the lesion increases in severity, the number of macrophages increases while the DCs decrease. The T cells are decreased in advanced lesions, but the cells that are affected are the CD4 while CD8 remain stable (Pers et al., 2012).

1.4.2.2 Cytokine production

Cytokines are produced by a broad range of cells and a given cytokine can be produced by more than one cell type with a pleiotropic effect, an example of some cells is provided:

Activated T lymphocytes (CD4 mainly) with high expression of HLA class II molecules and with the presence of co-stimulatory molecules, will differentiate into T helper (Th) 1 that produces IL-2, -3, IFN- γ and TNF- α promoting cell mediated response and to Th2 that produce IL-4, IL-5, IL-6, IL-10 and IL-13 which is engaged in humoral response. Th1/Th2 cross regulate (balance) each other by their cytokines secretions (Pers et al., 2012). Conflicting studies describing the role of regulatory T cells in the pathogenesis of SS in animals and patients were reported. Several studies have indicated that even though Th1 responses are thought to predominate in SS autoimmune lesions, evidence suggest that Th2 are the leading cytokines in mild lesions and re-emerging later in advanced lesions. In addition, with the discovery of IL23, identification of a new subset of Th cells which is Th 17 (secretes IL-17, IL-22 and other pro-inflammatory cytokines) in patients' salivary glands and bloods was done indicating a high correlation with SS pathogenesis and warranting further investigation. Activated B- cells may differentiate to produce similar Th1 and Th2 cytokines as well; Be1 and Be2. These cytokines maybe classified to pro-inflammatory, immunosuppressive cytokines and hematopoietic growth factors in addition to BAFF production (Pers et al., 2012).

Macrophages are one of the important components of SS histological lesions; they produce IL-18 that was found associated with higher risk of lymphoma development. While IL-12 was found associated with lower risk of lymphoma development, both of them are produced by DC cells as well (Manoussakis et al., 2007). Other cells include epithelial cells as mentioned previously and various stromal cells.

When dividing the cytokines into Th1 Th2 or any other cells one must bear in mind that although mentioned above as being produced from and exhibiting a certain function, they might be produced from other sources with different function; an example of which is IL-6 pro-inflammatory cytokine produced by different cells as monocytes/macrophages and DCs, and by T cells in chronic inflammatory conditions enhancing lymphocytes B growth and maturation. Also, it seems to be important for resolving acute inflammation, by inducing mechanisms that will shift innate to adaptive responses (anti-inflammatory). Lastly, IL-6 has also been shown to be involved in Th17 cell activation (Sverremark-Ekstrom, 2010). This necessitates reporting the cytokine sources and functions with caution. Also, it must be not forgotten that chemokines are the motivating force of the lymphocyte recruitment. Initially pro-inflammatory chemokines such as CXCL10 (IP-10; IFN-gamma-inducible protein) are involved, while at advance stages the homeostatic chemokines CXCL12, CXCL13, macrophage inflammatory protein-3- β (MIP-3- β); (CCL19) and secondary lymphoid-tissue chemokine (SLC); (CCL21) play an important role in inflammatory infiltrate maintenance and progression (Bombardieri and Pitzalis, 2012, Kroese et al., 2014, Barone et al., 2008). **Tables 1-5 and 1-6** summarise most of the important cytokines/chemokines with their functions adapted from (Murphy and Weaver, 2016).

Table 1-5 Cytokines and their functions

Family	Cytokines	Producer cells	Function
Interferons	IFN- α	Leukocytes, Dendritic cell	Antiviral, Increased MHC class 1 expression
	IFN- β	Fibroblasts	Antiviral, Increased MHC class 1 expression
	IFN- γ	T cells, NK cells, neutrophils, intraepithelial lymphocytes	Macrophages activation, increased expression of MHC molecules and antigen processing components, Ig class switching and suppress Th17 Th2
Interleukins	IL-1 α	Macrophages and epithelial cells	T cell and macrophages activation and fever
	IL-1 β	Macrophages and epithelial cells	T cell and macrophages activation and fever
	IL-2	T cells	T cell proliferation and differentiation, T reg maintenance and function (T cell growth factor)
	IL-3	T cells, thymic epithelial and stromal cells	Synergistic action in early hematopoiesis
	IL-4	T cells and Mast cells	B cell activation, IgE switch, induce differentiation into Th2 cells
	IL-5	T cells and Mast cells	Eosinophil growth and differentiation
	IL-6	T and B cells macrophages and endothelial cells	T and B cell growth and differentiation, acute phase protein production and fever
	IL-10	T and B cells macrophages and dendritic cells	Potent suppressor of macrophages function (cytokine synthesis inhibitory factor)
	IL-12	Macrophages and dendritic cells	Activates NK cells, induces CD4 T cell differentiation into Th1 like cells (NK- cell stimulatory factor)
	IL-13	T cells	B cell growth and differentiation, inhibit macrophage inflammatory cytokine production and Th1 cell induces allergy/asthma

Table 1-5 (Continued)

Family	Cytokines	Producer cells	Function
Interleukins	IL-17A	Th 17, CD8 T cells, NK cells and neutrophils	Induce cytokine and antimicrobial peptide production by epithelia, endothelia and fibroblast (pro-inflammatory)
	IL-18	Activated macrophages and kupffer cells	Induce IFN- γ production by T cells and NK cells and promotes Th1 induction
	IL-21	Th2 cells, T cell and primarily T _{fh} cells	Germinal centre maintained, induce proliferation of T, B and NK cells
	IL-22	NK cells TH17 and Th22 cells neutrophils	Induce cytokine and antimicrobial peptide production, induce liver acute phase proteins, pro-inflammatory agents and; epithelial barrier
	IL-23	Dendritic and macrophages	Induce proliferation of Th17 memory T cells increase IFN- γ production
	IL-33	High endothelial venules, smooth muscle and epithelial cells	Induces T _{h2} cytokines (IL-4, -5 and -13)
TNF	TNF- α	Macrophages, NK and T cells	Promotes inflammations, and endothelial activation
	APRIL	Activated T cells	B cell proliferation
	BAFF	B cells pDC	B cell proliferation

MHC; major histocompatibility complex, TNF; tumour necrosis factor, APRIL; a proliferation-inducing ligand, BAFF; B-cell activating factor, NK; natural killer cells, T_{fh}; follicular T helper cells.

Adapted from Murphy and Weaver (2016)

Table 1-6 Chemokines and their target cells

Chemokines	Name	Target cells
CXCL8	IL-8	Neutrophil, basophil CD8 subset and endothelial cell
CXCL10	IP-10	Activated T cells (Th1> Th2), NK cells B and endothelial cells
CXCL11	I-TAC	Activated T cells (Th1> Th2), NK cells B and endothelial cells
CXCL12	SDF-1 α/β	CD34 bone marrow cell, thymocyte, monocyte/macrophages, naïve activated T cell, B cell, neutrophil, plasmacytoid, immature and mature dendritic cell
CCL2	MCP-1	T cells (Th1> Th2), monocyte, basophils immature dendritic cell and NK cell
CCL3	MIP-1 α	monocyte/macrophages, T cells (Th1> Th2), NK cell, basophils, immature dendritic cell, Eosinophil, neutrophil, astrocyte, fibroblast and osteoclast
CCL4	MIP-1 β	monocyte/macrophages, T cells (Th1> Th2), NK cell, basophils, immature dendritic cell, Eosinophil, B cell
CCL5	RANTS	monocyte/macrophages, T cells (memory T cell> T cell; Th1> Th2), NK cell, basophils, immature dendritic cell, Eosinophil

Table 1-6 (Continued)

Chemokines	Name	Target cells
CCL7	MCP-3	T cells (Th1> Th2), monocytes eosinophil, basophils, immature dendritic cell and NK cells
CCL19	MIP-3 β	Naïve T cells, mature dendritic cell and B cells
CCL20	MIP-3 α	T cells (memory T cells, Th17 cells) blood mononuclear cells, immature dendritic cells, activated B cells NKT cells GALT development
CCL21	SLC	Naïve T cells, B cells, thymocytes NK cells and mature dendritic cell and

NK; natural killer cells, IP-10; IFN-gamma-inducible protein 10, I-TAC; Interferon-inducible T-cell alpha chemoattractant, SDF-1; stromal cell-derived factor- 1, MCP-1; monocyte chemoattractant protein-1, MIP-1 α ; macrophage inflammatory protein 1-alpha, RANTES; regulated on activation, normal T cell expressed and secreted, SLC; secondary lymphoid-tissue chemokine.

Adapted from Murphy and Weaver (2016).

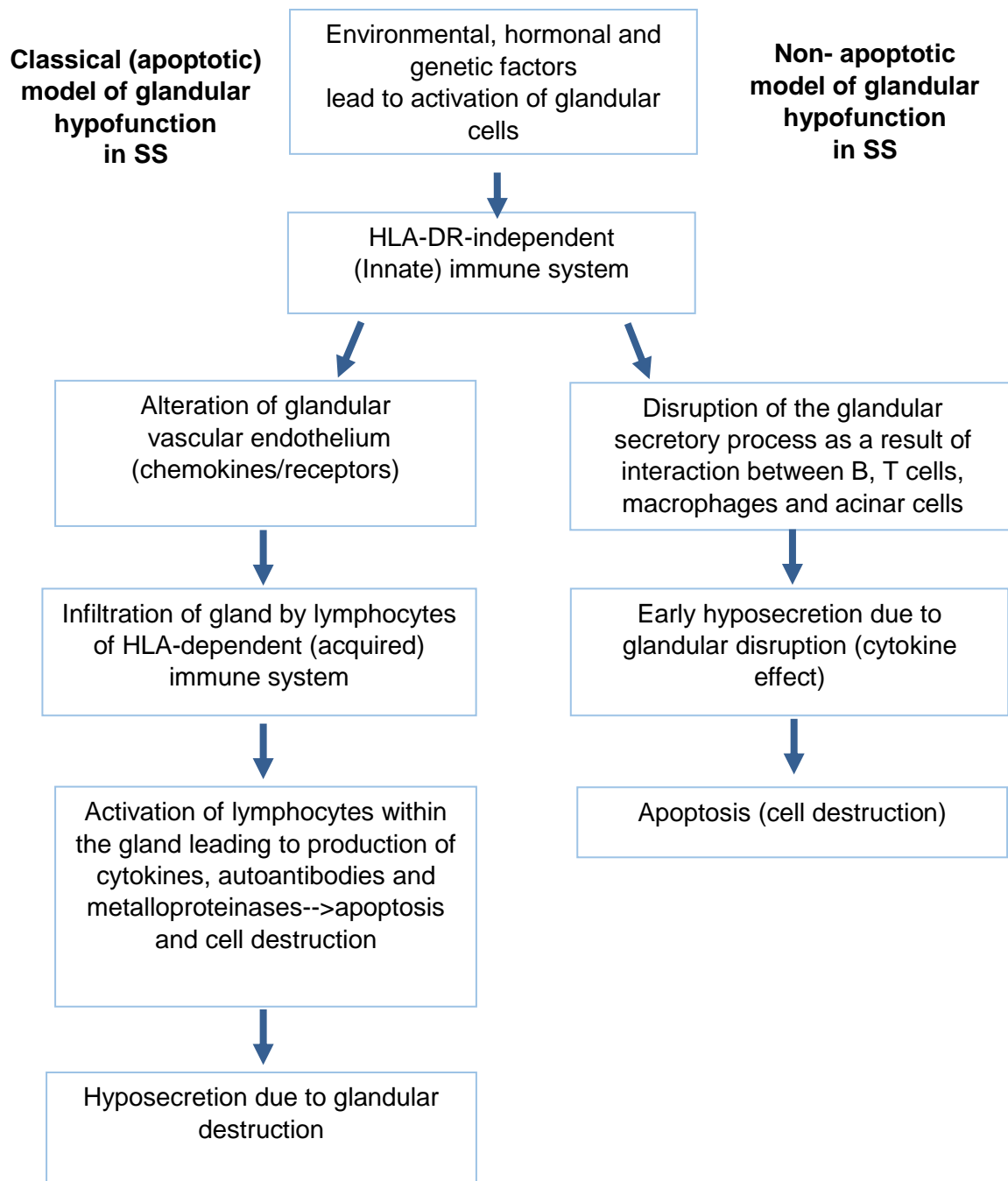
Cytokines in general contribute to the pathogenesis of SS in various ways; play a vital role in the initiation and continuation of inflammation in the secretory glands, may have a direct effect on epithelial cells, linked to systemic complications of SS and may play a role in lymphomagenesis as well. While the pathogenesis of SS remains unclear, evidence suggests that cytokines play a central role in SS;

- Elevated mRNA expressions of inflammatory cytokines have been consistently shown in affected tissues (minor and major glands).
- Cellular studies have verified that cytokine-induced apoptosis of secretory epithelial cells leading to secretory dysfunction.
- Studies on cytokine gene animal models have stated that both Th-1 and Th-2 cytokines are important for SS development.
- Studies reporting over expression of cytokines in serum, saliva and even tear of SS patients compared to controls (Roescher et al., 2009).

Roescher et al., (2009) provided a substantial review on the commonly identified cytokines in SS, some of the studies are summarised in (**Appendix 20-24**) while a summary is provided in (**Appendix 25**) as part of the review of the literature which is used as part of the methodology applied for chapter 5 with the addition of recently reported studies.

The aetiology and pathogenesis of SS are still far from being understood. Despite continued extensive studies, several issues are yet to be addressed. Given the important role of cytokines in SS, they were investigated in (chapter 5). Obviously, more studies are needed to understand the complexity of such disease. An overview of the pathogenesis of SS is present in **figure 1-5**.

Figure 1-5 Overview of pathogenesis of SS



Modified from Fox, (2005) and Dawson et al., (2006).

1.5 Prognosis of Sjögren's syndrome

In most cases SS has a good prognosis in respect to mortality as it is a chronic slowly progressing disease characterised by sicca symptoms with a generalised pain and fatigue. Some patients may exhibit extra-glandular manifestation which might be life threatening and may exhibit some of the considered risk factors of lymphoma development which is one of the most serious manifestations in SS. Median time for development of lymphoma after the diagnosis of SS has been reported to be about 7.5 years and a median age of 58 (Voulgarelis et al., 2012). While more recently a median age of 55 with a median time of 5.3 years from time of diagnosis was reported (Papageorgiou et al., 2015b).

The suggested three stages of SS progression are:

- Initial glandular infiltration of CD4+ Th cells and then B cells with the presence of serum autoantibodies.
- Infiltration of lymphocytes into the extra-glandular tissue and the development of pseudolymphoma and hypergammaglobulinaemia.
- Progression to B cell lymphoma, possibly attended by hypogammaglobulinaemia and immunodeficiency; hence, it has been suggested that SS is a lymphoproliferative disorder (Moriyama et al., 2012).

The following section will be summarizing the important aspects of lymphoma development in SS.

1.6 Development of mucosa associated lymphoid tissue lymphoma (MALT-L) in Sjögren's syndrome

1.6.1 Background

1.6.1.1 Historical perspective of MALT-L

The association between SS and the subsequent development of malignant lymphoma was first described in 1963 (Bunim and talal, 1963). 20 years later, the concept of mucosa associated lymphoid tissue lymphoma was introduced by Isaacson and Wright, (1983) where they described 2 cases of gastrointestinal lymphoma with a histology resembling that of MALT rather than lymph nodes, a year later a description of lymphoma in extra-intestinal sites with a similar histology was reported (Isaacson and Wright, 1984).

Following on from this, MALT lymphomas became recognised as a distinct subtype of NHL; several classifications have been proposed, first recognition of MALT lymphoma as a discrete entity was in 1994 by The Revised European- American Lymphoma (REAL) classification, it was classified under the group of marginal zone B- cell lymphoma (MZLs) (Harris et al., 1994). Three subtypes of MZLs were identified in the more recent criteria by the World Health Organisation (WHO) for lymphoma, (established in 2001 and updated in 2008); nodal, extra-nodal (MALT-L) and splenic type. This classification has shown to be more precise than any of the previous classifications and it has since become used by most pathologists and clinicians worldwide (Jaffe, 2009).

1.6.1.2 Types

Generally, MALT-L can develop nearly in every organ and it is divided to either gastric (most common) or non- gastric MALT-L; head and neck region is the second most common site where tonsil and salivary gland are commonly involved amongst others. Salivary gland MALT-L is almost always associated with SS; if there is a prolonged antigen-driven stimulation triggering subsequent lymphoid proliferation a malignant clone can emerge and a MALT-L can develop. MALT-L are low grade lymphomas and can occur in any of the salivary glands, but the parotid gland is most commonly involved in addition to other MALT sites that might be involved. According to the WHO classification the official name used to be extra-nodal marginal zone B- cell lymphoma, the only modification done later in 2008 was removing the term B-cell (Cohen et al., 2006). There were other types of lymphomas that have been reported with SS, but MALT-L followed with diffuse large B-cell lymphoma (DLBCL) are by far the most common types associated with SS (Anderson et al., 2009).

1.6.1.3 Epidemiology

Today, it has been known that patients with certain autoimmune and inflammatory disorders such as SS have an increased risk of developing malignant lymphoma. The risk varies between studies due to the heterogeneity of the sample sizes. Initially the risk was overestimated. Subsequently the standardised incidence ratio (SIR) was estimated at 20 and 15 according to Zintzaras et al., (2005) and Theander et al., (2006) respectively. While in a more recent study a lower ratio was estimated (SIR =7) (Weng et al., 2012). Furthermore, a study by Fallah et al., (2014) reported a standardised incidence ratio of 4.9. According to Nishishinyaetal., (2015), the risk was 4, 10 and 18% during the first 5, 15 and 20 years respectively. Parotid gland is the most commonly affected site displaying a 1000 fold increased risk (Zintzaras et al., 2005). It has usually a good prognosis with a 90.9 % overall survival rate (OS) (Papageorgiou et al., 2015b). 81% OS was reported in another study but lifelong follow-up is needed due to a high recurrence rate that reaches up to 35% over five years according to Anacak et al., (2012).

1.6.2 Pathological features and Histology

Clinically, salivary MALT lymphomas are localised (as an enlargement of the gland often unilateral and hard) and clinically it would be difficult to differentiate it from benign hyperplasia. No B symptoms are observed with rare involvement of the bone marrow. They exhibit a very indolent course with an extended progression time and a generally excellent prognosis, even without treatment. Some cases develop disseminated disease, usually to other MALT sites (ocular adnexa, stomach) or to lymph nodes. A few may transform to aggressive diffuse large B- cell lymphoma (Nocturne and Mariette, 2015). Para-neoplastic manifestations can include; vasculitis and neuropathy amongst others which might be extra-glandular manifestation of SS and used as predictive factors for lymphoma development as well which makes it difficult to differentiate between these 2 causes, and due to its indolent nature it is usually difficult to detect them (until later stages) (Risselada et al., 2013).

Histologically, most salivary gland MALT lymphomas occur on a background of myoepithelial sialadenitis (MESA); benign lymphoepithelial lesion. It is a key feature consisting of atrophic acinar tissue infiltrated by small lymphocytes and plasma cells, often with reactive lymphoid follicles. Typically, there are numerous epimyoeplithelial islands; which are often seen in major glands and might be seen in minor glands but not often and might be difficult to recognize because the glands are destroyed. The lymphoid tissue part consists of ectopic germinal centres while the epithelial islands consist of basal epithelial cells and modified myoepithelial cells with few ductal cells (**figure 1-6**). More advanced MALT lymphomas are characterised by a damaging, proliferations of neoplastic marginal zone B cells, “cavitating” lymphoepithelial lesions with a gradual replacement of reactive follicles. The neoplastic lymphoid cells are a distinctive and constant; these are centrocyte-like cells, in some cases and they might resemble monocytoid cells. Furthermore, scattered transformed blasts (large cells) and plasma cells are present as well (Bacon et al., 2007). It is difficult to differentiate between reactive follicle and neoplastic cells; but larger infiltrate, clear evidence of cytological atypia and monotypic lymphoid population are more indicative of lymphoma.

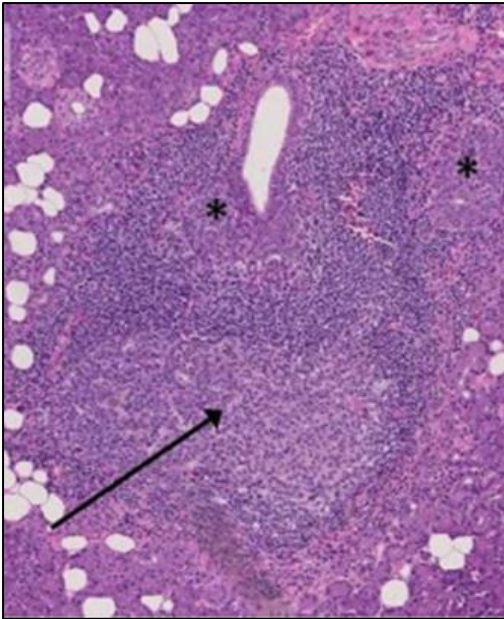


Figure 1-6 MALT- lymphoma of the parotid gland
Arrow indicates the germinal centres while asterisks indicate lymphoepithelial lesion.
Adapted from Delli et al.,(2016).

1.6.3 Lymphomagenesis of MALT-L

Emerging data connecting autoimmune disease to lymphoma development has been described (Mandal et al., 2014, Baecklund et al., 2013). Yet the exact mechanism is still far from being understood. The following is a short description of the transition from SS to lymphoma.

Continuing from the pathogenesis of SS and as mentioned previous; B- cell hyperactivity appears to be a hallmark of SS with transition of MESA to lymphoma in a multi-stage manner; with continuous stimulation and increased focal stimulation, ectopic, dysregulated germinal centres (GC) are likely to occur which are representing the lymphoepithelial lesions. The auto-reactive B- cells in these sites are associated with follicular DC networks resulting in the formation of memory B- cells at these sites. With sustained survival of B- cells and continuous production of autoantibodies, it would promote the expansion of unusual B-cell clones and produce the outgrowth of monoclonal aggregates of B cells. Increased B- cells clonal expansion in salivary glands of SS patients compared to controls was discussed in a review (Kroese et al., 2014). Neoplastic transformations of these clones are implicated for lymphoma development. It is still unclear what has driven the B- cells into clonal proliferation. Both ectopic GC and clonal expansion are associated with the risk of lymphoma development though the latter showed no absolute correlation (Jonsson et al., 2007). Yet there is a debate in the literature whether monoclonality can reliably diagnose MALT-L and differentiate it from MESA as it is difficult to differentiate between these entities histologically (Vega et al., 2005) (summary is provided in **figure 1-7**).

1.6.3.1 Possible mechanisms underlying the development of lymphomas

The following section is based on a review done by Dong et al., (2013). As it is an autoimmune disease with characteristic infiltrations of T and B lymphocytes, the following mechanisms or causes were suggested demonstrating the importance of interactions among T cells, B cells and epithelial cells:-

1.6.3.1.1 T cell dysregulation

T cells can:

- Stimulate B cells via the CD40-CD40L interaction with the action of various cytokines and chemokines.
- Stimulate the production of BAFF; may contribute to the lymphoma development.
- Allow clonal B lymphoid cells to escape immunological surveillance and elimination by inhibiting the protective T cell polyclonal lymphocytic infiltration into mucosal and exocrine tissues through regulatory T cells.
- Regulate and participate in the autoimmune pathogenic process (T follicular helper) cells.

Proliferation and differentiation of T cells are regulated by regulatory B cells which in turn if become dysregulated will affect the T cells dysregulating them as well. All of which may enhance the tendency toward lymphoma development.

1.6.3.1.2 Abnormal B cell biology

B cell accumulation is seen mainly in the later stages of SS supported by the following: -

- Reduction of circulating CD 27+ memory B cells while increased in inflamed tissue.
- Disturbed distributions of peripheral blood B cells classification which may enhance lymphoma development.
- Over expression of both BAFF and APRIL contributing to sustained survival of auto-reactive B cells which consequently lead to lymphoma development.
- Aberrant genetic mutations will generate B cells with defective apoptosis favoring lymphoma development.

- Stimulation of RF-producing clones via immunoglobulins which are important in the development and expansion of MESA-associated clones as well.
- Expression of hyper-mutated immunoglobulins with RF properties.
- Clonally expanded B cells in salivary glands may be at high risk of lymphoma development.

1.6.3.1.3 Cytokines

- Both Th1 and Th2 cytokines are implicated in SS development with shift toward the Th1 in the later stages then re-emergence of Th2 in lymphoma development.
- BAFF over expression is controlled by type I IFN leading to lymphoma development.
- Increased Levels of Fms-like tyrosine kinase 3 ligand (FLT3L) which were correlated with advanced lesions and lymphoma development.
- Expression of chemokines CXCL13 and CCL21 in lymphoepithelial lesions while CXCL12 in lymphoma lesions indicating its importance.
- Involvement of IL-6 in rearrangement in immunoglobulin V region genes during B-cell proliferation.

Refer to **Appendices 20-25** for the relevant references.

1.6.3.1.4 Viruses

Several viruses have been linked to SS as mentioned before and it was hypothesized that they are not only implicated in initiating the immune response but act as a sustained antigen leading to sustained B cell proliferation and consequently to lymphoma development. One study found no relation between lymphoma development and EBV, CMV, HIV and H. Pylori infection even though they were linked with other MALT lymphomas (Solans-Laqué et al., 2011). Some studies have found an association with HCV, a lymphotropic virus that mainly affects B cells. However, this association remains uncertain. Furthermore, another link to Human herpes virus infection was reported in a review.

Moreover, several oncogenic events, translocations and genetic polymorphisms have been reported to facilitate lymphoma development (Dong et al., 2013).

1.6.3.2 Cellular and molecular pathways summary

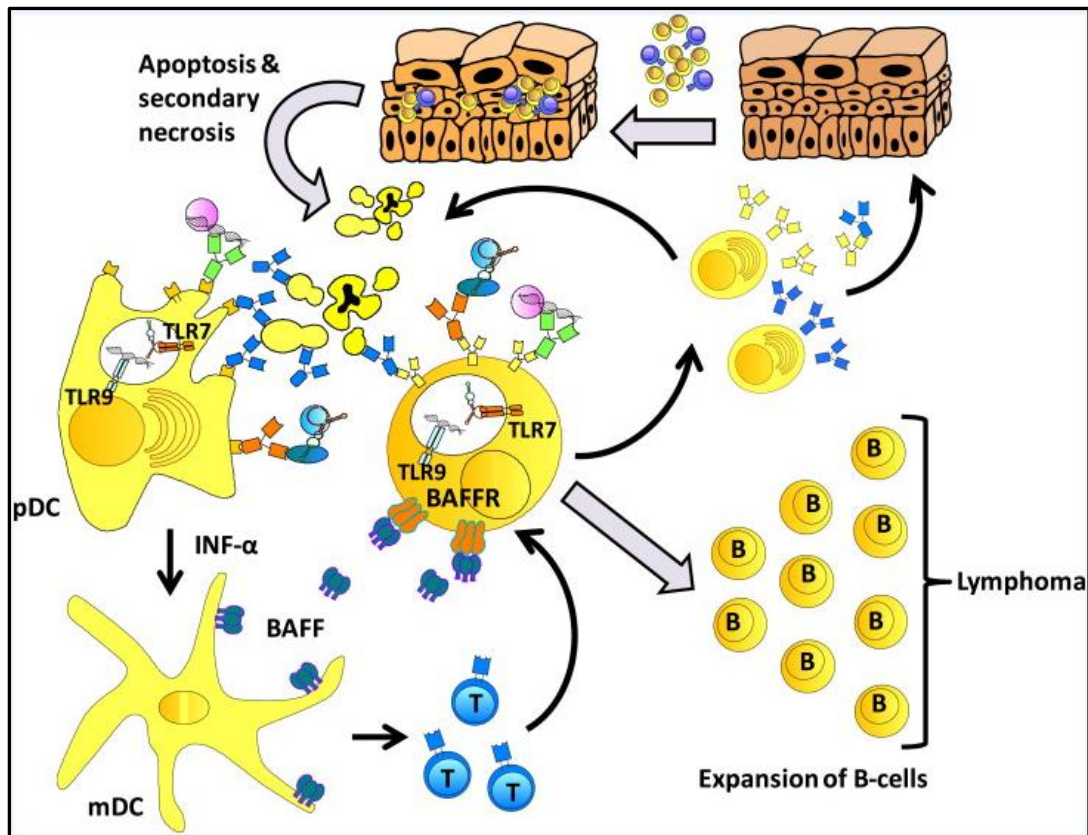


Figure 1-7 Cellular and molecular pathways implicated in lymphomagenesis in SS

Under certain conditions with the involvement of several factors, salivary gland epithelial cells are believed to start activated and deregulated initiating the inflammatory process and recruiting the lymphocytic infiltrate; with the production of pro-inflammatory cytokines they are further activated. Accordingly, apoptotic or/and necrotic epithelial cells will develop due to the immune attack releasing autoantigens or non- apoptotic cell will release exosomes containing these autoantigens complexes. These complexes will activate the plasmacytoid dendritic cells (pDC) through TLR producing INF-α which in turn facilitate the production of BAFF by the myeloid dendritic cells (mDC). Over production of BAFF will lead to aberrant B cell maturation with the production of autoreactive B cells that produce autoantibodies and rheumatoid factors and this long-lasting activation of innate and adaptive immunity will lead to immunologic exhaustion whereby the polyclonal expansions is switched to oligoclonal then finally to monoclonal cells with surface immunoglobulins displaying RF-like activity and a malignant clone may emerge that might ultimately lead to lymphoma development.

BAFF; B-cell activating factor, BAFFR; BAFF receptor, IFN-α; interferon-α.

Adapted from Routsias et al.,(2013).

1.6.4 Predictive risk factors

Despite efforts to identify risk factors for lymphoma development, neither the onset of its development can be identified efficiently nor which patient will develop lymphoma. Some predictors have been repeatedly reported by a number of studies with no clear explanation as to why they are linked to lymphoma development, however they share the same biological rationale, being a multistep disease that has antigen- driven stimulation, and so it will transform polyclonal B cells to neoplastic cells through a benign B cell clone. According to a recent review by Papageorgiou et al., (2015a) and a meta-analysis (18 published studies) by Nishishinya et al., (2015), a number of local and systemic disease related risk factors have been stated, some were well established while others were recently introduced **table 1-7**. Among the clinical predictors are parotid gland enlargement and skin vasculitides (specifically cryoglobulinemic type). Although the former failed to reach significance in some articles while the latter showed significance in few studies (Nishishinya et al., 2015). Nevertheless they were the most commonly reported predictors (Papageorgiou et al., 2015a). Lymphadenopathy was reported as significant predictive marker and lesser commonly stated predictors were splenomegaly, neuropathy and glomerulonephritis (Papageorgiou et al., 2015a, Nishishinya et al., 2015).

Regarding the serological predictors, the role of CD4 T cell lymphocytopenia has been confirmed as predictors and linked to lymphoma development in a number of studies, as well as a lower CD4/CD8 ratio. Few have regarded neutropenia as an independent risk factor. Several studies have reported mixed monoclonal cryoglobulinemia as a strong indicator of lymphoma, and one of them has associated it with advanced stages of MESA. While another study correlated MALT-L development with lower complement C4 levels which is another strong predictor stated by others as well, whereas C3 levels was found associated in some studies (n=3) but not in others (n=4). Hypergammaglobulinemia, β 2 microglobulin, anti- Ro /anti- La, and serum-free light chains levels have been linked with high disease activity but not lymphoma (Papageorgiou et al., 2015a, Nishishinya et al., 2015). Male-sex, anaemia, positive

ANAs and RF were not associated with lymphoma development (Nishishinya et al., 2015). A recent study demonstrated the link between peripheral neuropathy and lymphoma with low vitamin D levels. In addition, the role of soluble biomarkers like (s) CD22, (s) CD27 are yet to be investigated. However disturbance in the peripheral B cell subpopulations was linked to lymphoma with reduction of memory B cells in the peripheral blood and increased (s) CD27 (Papageorgiou et al., 2015a). Furthermore, high serum BAFF levels and FLT3 ligand levels were associated with lymphoma development and high disease activity (Papageorgiou et al., 2015a, Nishishinya et al., 2015)

With regards to histological predictors a lymphocytic focus score of 3 or more was correlated with lymphoma development. Also, the detection of ectopic GC-like structures was reported. Although they are still ill defined, and little is known about the mechanisms initiating their formation and potential subsequent lymphomagenesis, they are still considered one of the strongest risk factors as they are not linked to lymphoma only but with the disease activity as well. (Papageorgiou et al., 2015a). Given their importance, there is a need for consensus guidelines to standardise the evaluation of ectopic lymphoid infiltrates and GCs in salivary gland tissue of patients with SS (Delli et al., 2016). Regarding the evidence of monoclonality in tissues controversial studies were reported (Vega et al., 2005). Certain chemokines and cytokines were linked to lymphoma development as mentioned previously; CXCL12 expression was found in malignant lesions whereas CXCL13 and CCL21 were found in the reactive follicles (Barone et al., 2008). Expression of IL-12 and IL-18 were negatively and positively correlated with lymphoma development (Manoussakis et al., 2007). Also, several genetic factors were linked to lymphoma development such as the p53 mutation, BAFF and A20 genetic variations (Papageorgiou et al., 2015a). Furthermore, we have considered USS scores with the presence of hypoechoic foci as one of the risk factors as well and this will be discussed in chapter 3.

Table 1-7 Risk factors associated with lymphoma development in SS

Risk factors
Clinical
Recurrent or constant swelling of parotid glands *
Glomerulonephritis
Peripheral neuropathy
Skin vasculitis or palpable purpura*
Lymphadenopathy*
Splenomegaly
Serological/ haematological
Low complement factor C4 *
Mixed monoclonal cryoglobulinaemia*
Vitamin D serum levels
Neutropenia
CD4 T cell lymphocytopenia
Serum BAFF levels
Serum FLT3 ligand levels
Peripheral B cell subpopulation disturbance
Histological
High focus score ≥ 3
Germinal centre-like structures in minor salivary gland biopsies/ AID expression
CXCL12 expression
IL-18/IL-12 expression, dendritic cells infiltration rate
Genetic
A20 genetic variation
BAFF genetic variation
P53 mutations

BAFF; B-cell activating factor, FLT3 ligand; Fms-related tyrosine kinase 3 ligand.

* commonly reported.

Modified from Papageorgiou et al., (2015a) and Nishishinya et al., (2015).

1.7 Outcome and management

MALT- lymphoma prognosis is closely related to its diagnosis, the histological type of lesion and the stage of it; better prognosis for low grade lymphomas in comparison to high grade. No standardised protocols for lymphoma screening and follow up of patients with SS have been described and no ideal therapeutic strategy is defined yet. Treatment for each patient should be tailored depending on the severity of the disease. Currently, SS treatment mainly relies on symptomatic relief; analgesics, tears and saliva substitutes, sialogogues such as pilocarpine and cevimeline. Immunosuppressant and systemic steroids are prescribed for patients with extra-glandular manifestations however, their efficacy is yet to be determined. Among the B cell targeted therapy; rituximab was the most studied, though its efficacy is not enough to allow prescribing it in a large population (Cornec et al., 2014).

SS patients with known risk factors for lymphoma development (**table 1-7**) should be closely monitored. Patients with localised lymphoma can be managed by local radiotherapy or surgery and in the past the latter was preferred mostly. In addition, a more conservative watch and wait strategy for those localised lymphomas has been used by some centres since it has shown that treated and untreated patients have comparable favourable survival rates (87-95%) (Pollard et al., 2011). Advanced stage lymphoma may be treated with combination chemotherapy. A number of new promising drug candidates were introduced for the treatment of SS and lymphoma and some biological treatments have proven ineffective to control the disease. Furthermore no treatment will prevent its recurrence thus periodic local and systemic evaluation are needed during follow-up visits (Jonsson et al., 2012, Nocturne and Mariette, 2015).

1.8 Saliva

Saliva is produced by salivary glands that exhibit similar functional anatomy across all gland types with myoepithelial cells covering the acinar cells and a ductal system allowing saliva to pass to the oral cavity. The secretory end pieces or acini are composed of either major mucous (mucin) or serous (non-mucin) secreting acinar cells or a mixture of the two (muco-serous) (Tandler, 1993, Proctor, 2016).

1.8.1 Saliva composition

Saliva in humans is a dynamic fluid. It retains several important functions for maintaining oral health and homeostasis. The average composition of a sample of whole saliva is usually 99% water and less than 1% solids (macromolecules) (inorganic and organic components). The relative contribution of each gland is approximately 65%, 23%, 8%, 4% for submandibular, parotid, minor salivary glands and sublingual glands respectively in case of unstimulated saliva. While parotid glands contribute the most in case of stimulated saliva. Parotid glands are the source of serous saliva while the rest contains mix serous and mucous being the source of mucins. Also, it has non- glandular components from oropharyngeal mucosae. It may contain gingival crevicular fluid (GCF), food debris and blood derived compounds such as plasmatic proteins, erythrocytes and leukocytes (Chiappin et al., 2007). The inorganic components are ions (Na^+ , Cl^- , K^+ , Mg^{2+} , Ca^{2+} , HCO_3^- , HPO_3^-) present at different concentrations than in plasma. Furthermore secretions from different glands have different ionic levels (Chiappin et al., 2007). The organic components include: -

a) Non- protein components which include DNA, RNA and small amounts of uric acid, bilirubin, amino acids, cholesterol, fatty acids and glucose (Chiappin et al., 2007).

b) Proteins: such as amylase, cystatins, calgranulin, mucins and others, there are 2340 proteins identified (salivary or systemic origin) (Aframian et al., 2013). Protein secretion depends on the stimulation condition of the salivary gland with most proteins secreted by acinar cells. Among those for example are mucins (Muc5b and Muc7 gene products) secreted by the submandibular and sublingual glands as well as most minor glands but are not secreted

by the parotid and von Ebner's glands (Carpenter, 2013). The concentration of salivary components is flow rate dependent noting that they may originate entirely from the salivary glands or may be derived from the blood by passive diffusion or active transport. In the case of salivary cytokines, concentrations were reported to be higher in whole mouth saliva than in major glands having GCF as a primary source (Ruhl et al., 2004). However, the main sources in glandular tissue would be infiltrating cells, epithelial cells, endothelial cells amongst others.

1.8.2 Salivary gland physiology

Normal secretion of saliva requires an intact acinar and ductal anatomical structure with normal functioning acinar and ductal cells, intact neural stimulation to these cells as well as an intact vascular supply. Any factors interfering with these structures might lead to hyposalivation. Salivary secretion starts with an initial secretion of isotonic fluid by acinar cells which is then modified by the ductal system producing a hypotonic fluid which enters the oral cavity. Major salivary glands receive both parasympathetic and sympathetic nerve supplies, where the secretion is mainly controlled by the parasympathetic stimulation and to a lesser degree by sympathetic stimulation which differs in its intensity between the glands. It was reported that the parotid glands are supplied with fewer sympathetic nerves than the submandibular glands, and the labial minor glands are thought to lack a sympathetic secretory innervation. The salivary reflex is influenced by impulses from the higher centres of the central nervous system acting on the salivary centres in the medulla. The blood supply to the parotid gland is from branches of the external carotid artery (superficial temporal and maxillary arteries) and the venous drainage is via the retromandibular veins. Submental and sublingual arteries supply both submandibular and sublingual glands and the facial artery supplies the submandibular gland while the venous drainage is via anterior facial vein. The location of minor salivary glands in the oral cavity region itself determines the blood supply and venous drainage, reviewed by Proctor (2016).

1.8.3 Salivary flow

The average salivary flow varies between 0.5-1.5L per day at a rate of 0.4mL/min and approximately 50% is unstimulated flow, while the stimulated saliva contributes around 40% of the average daily production. The accepted normal range of unstimulated salivary flow is 0.1- 0.5 mL/min. Stimulated flow rate is almost 2 mL/min (Sreebny and Vissink, 2010). Unstimulated parotid saliva range is 0.03-0.05 mL/min while the submandibular is 0.04-0.06 mL/min. The stimulated glandular saliva usually is 3 times the unstimulated secretion that can reach up to 7 mL/min for parotid saliva and less than 0.2 mL/min is considered hyposalivation (Mandel, 1980). Nevertheless, general agreement about these cut-off values does not exist. Several factors can influence flow rate and composition (e.g. circadian rhythms, degree of hydration, body position and others) (Edgar et al., 2004) (Dawes, 1987).

1.8.4 Functions of saliva

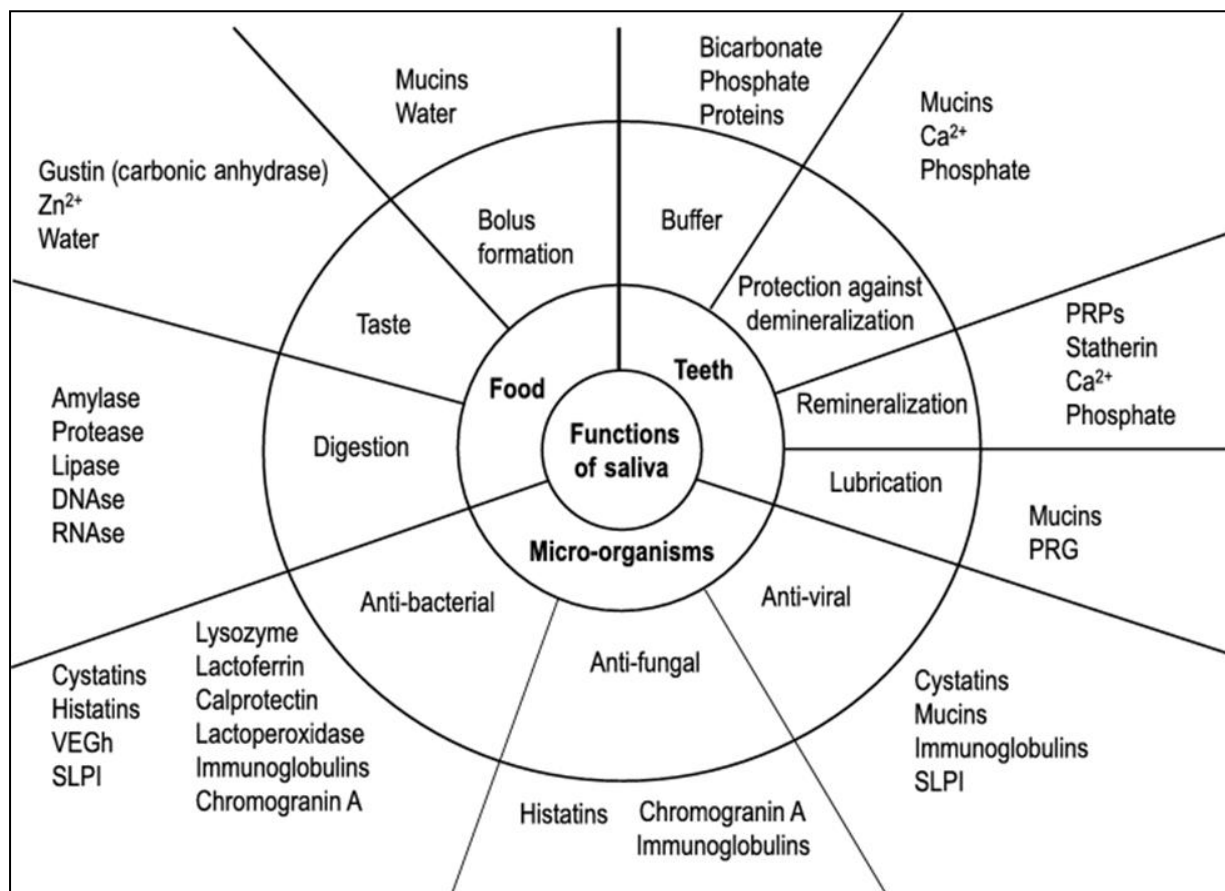


Figure 1-8 Main functions of saliva and its components (Amerongen and Veerman, 2002)

1.8.5 Saliva as a diagnostic tool

Saliva is gaining attention as an attractive alternative diagnostic tool, and is increasing in popularity. Collection of saliva is a non-invasive, safe, feasible, it doesn't require special training unless it is collected from a specific gland, and is a relatively easy process (Aframian et al., 2013). When collecting saliva samples, one should consider the effect of variability of flow rate for example intra- variation measurement error on repetition of 25% (Vissink et al., 2012). Also, have a standardised collection time and site, while unstimulated saliva collection is the preferable method but low volumes might be obtained especially with SS patients. A collection device is the best method for glandular specific saliva such as a parotid saliva sample. Sample storage conditions should also be optimised (on ice packs and frozen as soon as collected), samples should be handled consistently e.g. centrifugation and mucins effect on cytokines (Salimetrics)(discussed elsewhere).

Saliva can contribute significantly to disease screening, intervention evaluation, recurrence prediction, risk assessment, and other prognostic outcome measures. Its potential use as a diagnostic fluid depends on the ability to detect and stability of a named substance. For example, cytokines and chemokines might be inhibited differently by different types of saliva (being higher in whole mouth saliva than a specific gland sample). Also, it seems that the detection of immunoglobulins are not affected by the inhibitory effect of saliva and that might be explained by their volume difference (150 vs. 8-25 kDa) (Wozniak et al., 2002).

In the past decade, advances in mass spectrometric technologies led us to a new era in biomarker discovery. Salivary proteomics can enhance the sensitivity and specificity of disease detection, as the proteomics technologies continue to mature and expand allowing a possible identification of minute details; lower volume proteins such as cytokines. Detection of salivary biomarkers and the on-going progress of diagnostic technologies have addressed its diagnostic value for clinical uses.

Compared to blood, saliva may express more sensitive and specific markers for certain local oral diseases. Also, many blood components are reflected in saliva, which indicates that saliva

may offer a promising diagnostic tool for systemic disease. These components, if successfully found and validated they may serve as biomarkers, thus a non- invasive tool for diagnosis will be developed (Zhang et al., 2013). However, with respect to salivary composition, there is a wide range of variation, low concentration of diagnostic components and the possibility of blood contamination. This makes it difficult to develop standardized analytical techniques and this has prevented salivary diagnostics from being clinically practical, making blood sampling preferable. Also, one must bear in mind when analysing whole mouth saliva that it is influenced by the proportion of parotid to submandibular secretions as well as the effect of the non-salivary elements. In studies quantitating antigens, immunoglobulins, or any proteins which are not produced by the salivary glands, extreme care must be taken in examining whole saliva to eliminate gingival fluid especially if a whole saliva sample is collected with a masticatory stimulus (Mandel, 1980).

Saliva has been used mainly in dentistry as a tool for caries risk assessment (buffering capacity and bacterial count), periodontal disease assessment and for investigating systemic diseases which influence the oral cavity e.g. Behçet's disease and oral cancer. Some of the proposed diagnostic applications of saliva were; monitoring steroid hormone levels, detection of drug abuse and monitoring of some medications. It has been particularly useful for qualitative diagnosis (presence or absence of markers) such as viral infection detection (HIV, HCV and EBV diagnosis). Several studies have tried to find a link to oral, breast and pancreatic cancer using saliva; other studies have tried to use saliva for early detection of diabetes mellitus (Chiappin et al., 2007). In the case of SS and dry mouth in general, saliva has been used a part of routine diagnostic investigation, sialochemistry and proteomics have been done for SS. Cytokines linked with the inflammation in the gland could serve as saliva biomarkers in the diagnosis of SS and salivary gland lymphoma (Katsiougianis and Wong, 2016). (This will be discussed further in the relevant chapters).

When probing to identify disease-associated proteins, investigators tend to embrace a discovery approach (unravelling the whole proteome) and/or a targeted approach, whereby a selected number of proteins are further validated in a clinical setting. Each approach has its own merits and drawbacks. However, if proven beneficial, a saliva test could be a valuable supplement to established diagnostic methods. Due to the potential advantages of salivary analysis, more studies are needed. Consequently, we are likely to see it being used more often as a diagnostic tool and more people will be involved in the process of diagnosing a variety of disorders. What is becoming increasingly clear is that composition of saliva is much more complex and dynamic than earlier perceived.

1.9 Thesis aims

To enable a better understanding of disease activity and progression the following aims were undertaken:

1. Investigate the potential use of the salivary gland assessment tests in discriminating SS and non- SS sicca patients as well as to differentiate between the different subgroups of SS.
2. Determine the diagnostic accuracy of USS.
3. Complete a longitudinal study on dry mouth patients at 5 and 10-year time-points.

In chapter 3 records and assessment of the clinical parameters of salivary gland function was done. Some are commonly used in diagnosing Sjögren's syndrome patients such as whole and parotid flow rates while clinical oral dryness score is used as a supplementary test enabling clinicians to visualise the extent of hyposalivation. In addition, the ultrasound score has gained a considerable attention in the literature as an ideal non- invasive test thus determining its diagnostic accuracy was of great importance. The ability of the clinical parameters to stratify different subgroups of SS (SS at risk and SS with MALT-L) was looked at and their long-term outcome was considered. This is of particular relevance as salivary gland dysfunction is one of the main clinical features of SS, with its subsequent complications and its well-known effect on the person's quality of life thus monitoring an actual oral dryness is of significant importance. Furthermore, it was intriguing to compare between different subgroups of SS to identify whether these parameters can detect the differences between them with a special interest in ultrasound scores.

4. Identify salivary markers of disease activity and progression in SS; MALT-L risk and MALT-L subgroups.

In chapter 4 the proteomic profile of representative parotid saliva samples was determined and this was followed by a candidate marker selection and verification via ELISA where even matched whole mouth saliva was compared as well. The different subgroups will be included

together with SNOX and age, sex- matched healthy controls thus providing a new insight into the potential use of the candidate protein as a biomarker.

In chapter 5 cytokines were studied due to their significant role in several aspects of SS pathogenesis; their relevance in literature was recognized and gradual screening of cytokines was performed with a purpose of identification of their salivary levels via semi-quantitative tests. Then a more sensitive test (performance assay) was followed to further confirm their presence and their salivary levels. The same patients' groups (from chapter 4) were included to assess their ability in differentiating between these groups and their levels in whole mouth saliva were determined as well and compared. Finally, whether a relationship exists between selected salivary components (chapter 4 and 5 and the clinical parameters of salivary gland function (chapter 3) was determined.

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2 Materials and methods

2.1 Clinical

2.1.1 Ethical approval

The protocol for this study was reviewed and approved by the National Research Ethics Service (NRES) Committee London-Brent since October 2011, reference number: 11/LO/1121. All the participants signed an informed written consent form.

2.1.2 Study group

Patients with symptomatic dry mouth, attending clinics at Guy's and St. Thomas's Hospital Oral Medicine department (GSTT NHS Foundation Trust), were placed in the following diagnostic groups:

- SS with or without another autoimmune disease (AID).
- Sialadenitis, nodal osteoarthritis & xerostomia (SNOX).
- Drug induced hyposalivation (DIH).
- Not otherwise specified (NS) for patients who did not fit any of the above groups.

SS classification was based on the most widely accepted criteria that was proposed by the American-European Consensus Group in 2002 (Vitali et al., 2002). Early SS was not commonly included while patients with confirmed or suspected lymphoma attending Oral Medicine clinics and lymphoma clinics (Department of clinical oncology) were sub grouped accordingly to:

- SS at risk of developing MALT lymphoma; further categorised into high and low risk patients.
- SS /confirmed MALT lymphoma with no treatment
- SS /confirmed MALT lymphoma post-treatment

The diagnosis of lymphoma was histologically proven in all cases either following excision of the parotids, via fine needle aspiration (FNA), parotid or labial gland biopsy.

Healthy age and sex-matched controls were recruited from Oral Medicine, Oral Surgery, Undergraduate Care Planning clinics and amongst staff. Their salivary samples were used for chapters 4 and 5.

For chapter 3 all disease control sicca patients mentioned above were included while only SNOX was selected for further salivary analysis in order to verify its presence among dry mouth patients while comparing it to SS patients with a purpose of not adding variable groups in the disease control patients and focusing on the variability within the SS subgroups. Furthermore, considering the cost of including other samples it was decided to include only one group which was not previously been tested.

2.1.2.1 Exclusion criteria

- Salivary gland disease secondary to Hepatitis, HIV infection or other disease (Vitali et al., 2002).
- Infected gland with pus discharge which might interfere with analysis results.
- Patients with a provisional diagnosis of SS, who did not have their biopsies done and/or their autoantibodies levels measured were excluded.

It was made clear to all subjects that participation will be anonymised. Participants were given an outline of the project in an information sheet.

2.1.3 Data collection

Electronic patient records (EPR) and case files of patients were reviewed. Patient's demographics, dates of initial and last visits as well as their diagnosis were recorded. Clinical parameters including: Clinical Oral Dryness Score (CODS), Ultrasound Scores (USS), Salivary Flow Rates (parotid and whole mouth), labial Salivary Gland Biopsy results (LSG), and autoantibody levels (ANAs, ENAs and Rheumatoid factor) were all recorded and the information was entered into organized data sheets.

CODS also referred to as The Challacombe Scale enabling clinicians to visually identify and semi-quantify the amount of dryness with a score of 1-10 where 1-3 indicate mild dryness, 4-6 moderate and 7-10 indicate severe dryness (Challacombe et al., 2015) (**Appendix 1**).

To evaluate salivary gland changes on ultrasound imaging, Dr Jacqueline Brown Consultant Radiologist and calibrated team at Guy's and St. Thomas's Hospital, developed a simplified scoring system (Brown, 2010). It is based on a system proposed by Hočevár et al., (2005). The radiologists were calibrated and worked to an agreed pictorially-based and verbally descriptive scoring template. The score of 1 is the least involved while 9 is the severely involved. Patients with a score of >4 and bilateral glandular involvement were considered SS patients (**Appendix 2**).

Focal lymphocytic sialadenitis (FLS) is the characteristic pattern of inflammatory infiltrate seen in the glandular biopsies of SS patients and it is scored according to Chisholm and Mason Score. Focus Score= Number of lymphocytic aggregates (≥ 50 cells) per $4 \text{ mm}^2 \geq 1$ (Chisholm and Mason, 1968).

Data were recorded for the cross- sectional study where the 4 main parameters (whole and parotid flow rates), CODS and USS were compared between SS subgroups (SS, SS at risk of developing MALT-L and those with MALT-L) as well as the disease control group (DCT). Concurrently correlation between those parameters was done while correlating focus score and USS only. For the longitudinal study, 80 patients were followed over a period of 5 years

and the same 4 parameters were compared at baseline and 5-year time points. Sixteen patients were followed over 10 years and again their parameters were compared.

Simultaneously, Clinical and laboratory predictors of lymphoma described earlier by Nocturne and Mariette, (2015) were recorded when found. For this study, patients were considered at risk of developing lymphoma if they had at least three of these factors with a tendency towards a higher risk with the presence of parotid gland swelling, purpura, low serum C4 complement, cryoglobulinemia, high focus score and germinal centre in their biopsy. In addition, higher ultrasound scores with the presence of the hypo-echoic foci were also considered as one of the predictive factors and their role in predicting lymphoma will be studied.

Furthermore, data regarding patients with confirmed lymphoma was recorded as well and it included: type and site of the lymphoma and type of treatment received.

To address the realistic issue of missing data potentially emerging from the retrospective nature of this study, frequent communication (whenever applicable) was made with the patients themselves, to gather more information and to invite them to participate and their samples were collected and used when needed.

2.1.4 Saliva collection

As a part of the routine clinical assessment only unstimulated whole mouth saliva (UWMS) and stimulated parotid saliva (SPS) were collected. The former was collected by asking subjects to passively drool into a 20 mL universal sterile plastic tube for 10 minutes. While SPS was collected into another universal tube for 10 minutes. The collection was done using a Lashley cup (Lashley, Dec 1916) (**figure 2-1**) and stimulation of secretion with a 2% Citric acid solution. The tubes were pre-weighed and then weighed again after collection in order to calculate the salivary flow rates (detailed clinical protocol is found in **Appendix 4 & 5**). Samples were immediately placed on ice and transferred to the lab where they were processed by centrifugation at 10,000 rpm for 10 minutes (whole saliva). Parotid saliva was left without centrifugation at first, but subsequent experiments (e.g. Luminex assays) identified the importance of centrifugation for at least 5 minutes. Samples were split into a number of aliquots and stored at -80 °C until required. None of the aliquots were used more than once per assay. (Start date for collection August 2013. End date March 2016).



Figure 2-1 The Lashley cup for the collection of parotid saliva

(A) The cup consists of inner and outer circular chambers (rings). (B) The inner ring is placed over the orifice of Stensen's duct and connected to a plastic tube for the collection of saliva. (C) The outer ring is used for suction and is connected to a syringe which is used to create a negative pressure that maintains the cup in place without movement. Modified from http://www.medscape.com/viewarticle/719259_9

2.2 Laboratory analyses

2.2.1 Total protein concentration

The total protein concentration of samples (parotid and whole saliva) was determined using Bicinchoninic Acid assay (BCA assay) (Thermo Scientific, Rockford, Illinois, USA), according to manufacturer's instructions. A standard curve of absorbance vs. protein concentration was generated using Bovine serum albumin which was serially diluted. Samples were diluted 1:10 in ultra-high-quality water (UHQ.H₂O) and both standards and samples were run in duplicate and absorbance at 450 nm was measured using a Dynex Revelation® 4.24 spectrophotometer. The final total protein concentrations of the samples were determined considering the dilution factor and expressed as mg/mL.

2.2.2 Sample preparation and Gel Electrophoresis

Samples were analysed for protein content using Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE). Precast NuPAGE® Novex® 4-12% Bis-Tris Protein gels cassettes (Invitrogen) placed in XCell vertical electrophoresis unit (Invitrogen), were used to separate proteins under reducing conditions according to molecular weight. Samples were prepared as recommended by addition of NuPAGE® lithium dodecyl sulphate sample buffer (LDS, Invitrogen), 10% 0.5M dithiothreitol (DTT, x 10 stock) followed by heating at 100 °C for three minutes. A final sample volume of 20 µl was prepared. SDS- Running buffer was prepared by diluting it with 25 mL of NuPAGE® MES SDS running buffer and 475 mL UHQ.H₂O. Ten µL of the prepared sample and 5 µL of SeeBlue® Plus Pre-stained marker (Invitrogen) were loaded into the wells. Electrophoresis was performed at 125 mA and 200 volts constant for 35 minutes. This was followed by gel staining.

2.2.3 Coomassie Brilliant Blue, total protein stain

Separated protein bands were visualised by incubating gels with ready-made Coomassie Brilliant Blue R 250 (sigma) diluted in acetic acid (10 mL of stain with 15 mL of acetic acid) for 30 minutes, with gentle agitation followed by destaining of background with 10% (v/v) acetic acid in UHQ.H₂O overnight. Gels were scanned on a transmission bed scanner (Amersham, Biosciences) where their digital records were taken.

2.2.4 Liquid chromatography tandem mass spectrometry (LC-MS/MS) and database searching

Following SDS- PAGE of parotid saliva samples collected from age and sex match patients and controls, the sample lanes were divided into four sections prior to reduction, alkylation and digestion with trypsin (Roche Diagnostics GmbH, Mannheim, Germany) at 37 °C for two hours then overnight at room temperature. Digests (peptides) were extracted from the gel pieces and dried to completion by speed vac (Thermo Fisher Scientific). The lyophilised peptide mixture was resuspended in 50 mM ammonium bicarbonate (Sigma Chemicals) prior to loading onto a nano-LCII chromatography system (Thermo Fisher Scientific). Peptides were separated by reversed-phase chromatography, firstly by trapping column (C18-A1, 5mm, 2cm packing) then by analytical column (C18-A2, 3mm, 10cm packing; both Thermo Fisher Scientific) at a flow rate of 300 nL/min using a three-step gradient (5-40% Acetonitrile (ACN)/0.1% formic acid (FA); 40%-95% ACN/0.1% FA; 95%-5% ACN/0.1% FA) for 120 minutes.

Peptides were ionised by electrospray ionisation using an Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific) operating under Xcalibur v2.2. The instrument was programmed to acquire in automated data-dependent switching mode, selecting precursor ions based on their intensity, for sequencing by collision-induced dissociation using a Top 20 method. The MS/MS analyses were conducted using collision energy profiles that were chosen based on the mass-to-charge (m/z) and the charge state of the peptide. Raw mass spectrometry data were processed into peak lists using Proteome Discoverer v1.4 (thermo Fisher Scientific).

All LC-MS/MS data were searched using the MASCOT database search algorithm (Matrix Science, London, UK) against the human portion of the Uniprot protein database (uniprot_sprot_130220_database) to obtain peptide and protein identifications. MASCOT results were imported into Scaffold v2.2.06 (Proteome Software, Portland, OR, USA) and viewed.

2.2.5 Enzyme linked immunosorbent assay (ELISA)

Commercially available pre-coated plates (Quantikine ELISA Kit, R&D Systems, Minneapolis, MN), (cat. no. DS8900) for S100A8/S100A9 Heterodimer (calprotectin) were used for both whole (n=56) and parotid saliva (n=83). The samples were thawed and diluted at an optimised dilution factor of (1/500) (serial dilution D1=1:50, D2=1:10) for whole saliva and (1/200) (serial dilution D1=1:10, D2=1:20) for parotid saliva. Standards and samples were loaded in duplicate. Incubations were done at room temperature on a horizontal orbital microplate shaker. All materials were supplied with the kit and the manufacturer's instructions were followed.

Generally, the samples and standards were added to the plate and any analyte present will bind to the antibody (capture antibody). The plate was then washed to remove unbound proteins. A second horse radish peroxidase (HRP)-labelled antibody (detection antibody) was added, incubated and washed to remove any unbound materials. This was followed by the addition of Tetramethylbenzidine (TMB) substrate solution and a blue colour changes in proportion to the amount of analyte present in the sample (**figure 2-2**). Colour development was stopped and measured at 540nm with a 450nm reference absorbance (Dynex Revelation® 4.24; see **Appendix 6** for detailed protocols).

The accuracy of the results was tested separately. To check for matrix interference the samples were spiked with a known concentration equivalent to the first standard (40ng/mL) (1:2). Three samples were spiked, 1 whole saliva sample and 2 parotid saliva samples (all positive with a concentration comparable to the highest standard). The samples were tested in duplicates in 4 dilutions (neat spiked sample, 1:2, 1:4, 1:8).

A four-point standard curve was generated with the same dilutions and compared to spiked samples. Optical density was measured at 540 nm and 450 nm as mentioned previously. Recovery and linearity were then calculated.

Intra-assay precision was assessed using 3 samples of known concentration each tested 5 times on one plate. Inter-assay precision was assessed using 2 samples tested in 6 separate plates. Samples with high readings or zero values were retested in order to confirm their results.

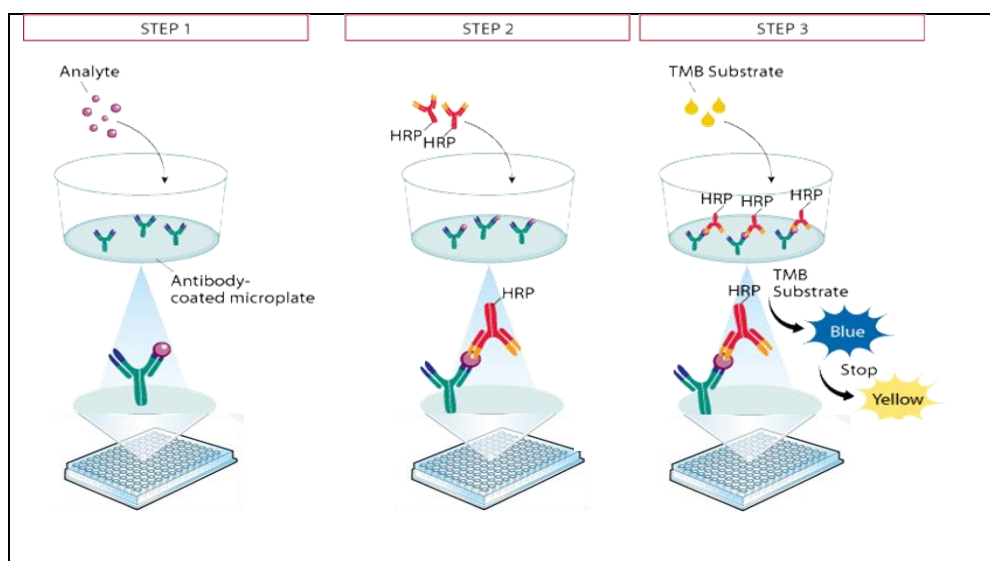


Figure 2-2 Enzyme linked immunoassay (ELISA) principle.

<https://www.rndsystems.com/products/quantikine-colorimetric-sandwich-elisa-assay-principle>

2.2.6 Proteome Profiler Array

The Human XL Cytokine Array (R&D Systems, Minneapolis, MN), (cat. no. ARY022) is a membrane-based sandwich immunoassay that enables the relative detection of 102 cytokines and chemokines simultaneously. The kit was used according to the manufacturer's protocol. All steps were carried out in a 4-well multi dish provided in the kit and the incubations were performed on a rocking platform shaker. In brief, all reagents and parotid saliva samples collected from subjects (four samples per kit) (n=12) were brought to room temperature. A suggested 100 μ L of saliva (range 50-200 μ L) was diluted with the array buffer (supplied with the kit) to a final volume of 1.5 mL. Then added to the membranes and incubated overnight at 4°C. These nitrocellulose membranes (used as a substrate rather than a plate in a traditional ELISA) are spotted with capture and control antibodies in duplicate, each pair of these spots represents a different analyte. The membranes were washed to remove unbound material followed by incubation with a cocktail of biotinylated detection antibodies. Streptavidin-horse radish peroxidase (HRP) and chemiluminescent detection reagents were then added, and a signal was produced at each capture spot equivalent to the amount of cytokine bound (**figure 2-3**). The chemiluminescence of the membrane was measured using the ChemiDoc (Bio-Rad, Hemel Hempstead, UK) then quantified by band densitometry using ImageJ software (downloaded from National Institute of Health, <https://imagej.nih.gov/ij/>). (Detailed steps are found in **Appendix 7**).

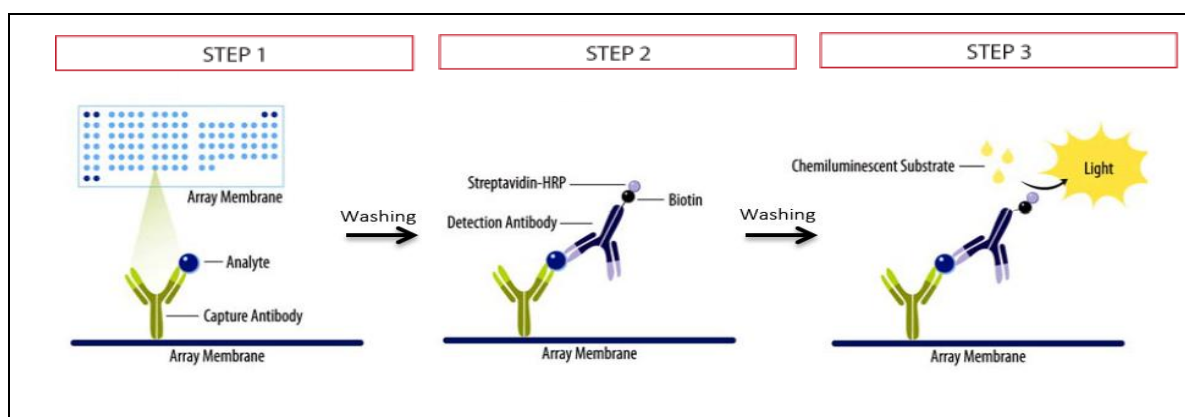


Figure 2-3 Membrane-based multiplex assay principle

<http://whitesci.co.za/brand/rnd-systems/proteome-profiler-antibody-arrays/>

2.2.7 Luminex® xMAP Assays

Bead- based multiplexed immunoassays (R&D Systems, Minneapolis, MN) allow the detection of multiple analytes in one assay and the main advantage of Luminex technology lies within its significant reductions in time and cost compared with ELISA, especially when many immunological markers needed to be measured and sample volumes are limited (Arellano-Garcia et al., 2008).

The principle of the assay is similar to ELISA: after the addition of samples and standards to the mixture of colour coded beads which are pre-coated with specific capture antibodies, the analyte of interest was bound to them. Biotinylated detection antibody cocktail was added and binds to the captured analytes, this is followed by the addition of Phycoerythrin (PE)-conjugated streptavidin which binds to the detection antibody cocktail. The assay was run on a 96-well plate format, followed by reading on a Luminex ® 100/200™ instrument (Bio-Plex®200). A magnet captures and holds the magnetic beads in a monolayer, while two spectrally separate light-emitting diodes (LEDs) lighten the beads. One LED recognizes the analyte that is being detected and, the second LED controls the amount of the PE-derived signal (**figure 2-4**). Images of the wells were taken with a CCD camera. Samples were assayed for analytes according to established protocols and incorporating standard curves, blank, controls (if present). The assays were completed in a minimum of 4 hours depending on the viscosity of the samples. Once readings were completed the results were exported to Excel spreadsheet. Account was taken of outlying sample results; present sample fluorescence –background (blank), standard deviation, coefficient of variance (%CV), recovery of analyte in sample, dilution, bead Count (minimum of 37) and sampling errors. Magnetic beads were selected and the screening assays were used at first in order to have more flexibility in creating the panel with a broader selection of analytes which can be up to 100 analytes simultaneously. Performance assays were performed subsequently on selected analytes for maximum assay precision and developing more accurate results and it can be up to 22 analytes per panel.

2.2.7.1 Magnetic Screening Luminex Assay

Twenty cytokines were selected and their levels were measured in parotid saliva of 76 subjects (detailed description of the groups is provided in the relevant chapter). The cytokines assayed included: IFN- γ , TNF- α , IL-1 α , -1 β , -2, -3, -4, -5, -6, -8, -10, -12p70, -17A, -22, -23, -33, IP10, MCP-1, MCP-3 and ST2.

The selection was based partially on the results obtained earlier from the proteome profiler analysis where substantial differences were observed between the groups. The remaining were cytokines in saliva (mainly whole) that were selected after conducting a thorough review of the literature which involved searching through the database for cytokines, SS and lymphoma (this will be addressed in chapter 5). Commercial kits for luminex assays optimized for detection of cytokines in serum, plasma and cell culture supernates were used following the manufacturer's instructions. Sample preparation and optimization was tested at first on a trial kit with a single panel with just two analytes (IL-17A and -23) where the minimum required dilution was determined by analysing multiple representative samples from each group at different dilutions (neat, 1:2, 1:4 and 1:8). Neat samples yielded the highest readings within the assay range thus no dilution was required. Also, the effect of centrifugation was assessed where it was noticed that lower bead count or bead aggregation occurred as a result of the viscous nature of the uncentrifuged samples. This was eliminated in most of the samples after centrifugation for few minutes. The analytes were provided in two customised panels (kits). One included IL-3 and ST2 (R&D Systems, Minneapolis, MN) (cat. no. LXSAHM-3), while the rest were included in the other panel (R&D Systems, Minneapolis, MN) (cat. no. LXSAHM-19). Standards and neat samples were added in duplicates and the steps were followed as described in the protocol (**Appendix 8**). Initially the standard curve was extended to <1pg/mL to further assess the sensitivity and the assay range. Points were excluded from the standard curve if the CV<25% and accuracy outside of 75-125% were not obtained, starting from the lowest standard. Samples still showing bead aggregation or bead count less than 37 were excluded (Staples et al., 2013).

2.2.7.2 Magnetic Performance Luminex Assay

Selected cytokines (IL-1 α , -1 β , -4, -6, -8 and MCP1) were measured in parotid saliva samples (n= 82) and whole saliva samples (n=57). The assays depend on panel-optimized diluents that provide maximum performance.

Again, a trial kit with a single panel (IL17, -23) was used in order to verify the minimum required volume of reagents to produce accurate results with ≥ 37 beads. Parotid samples that showed high levels of certain cytokines in the screening test were diluted (1:2), while whole mouth saliva were diluted (1:4) according to previous collaborative experiments by colleagues (S.Alsahaf thesis, KCL, 2015). A Single customised panel (cat. no. FCSTM03) was used to measure all of the six cytokines, using a similar protocol to the screening assay (**Appendix 8**).

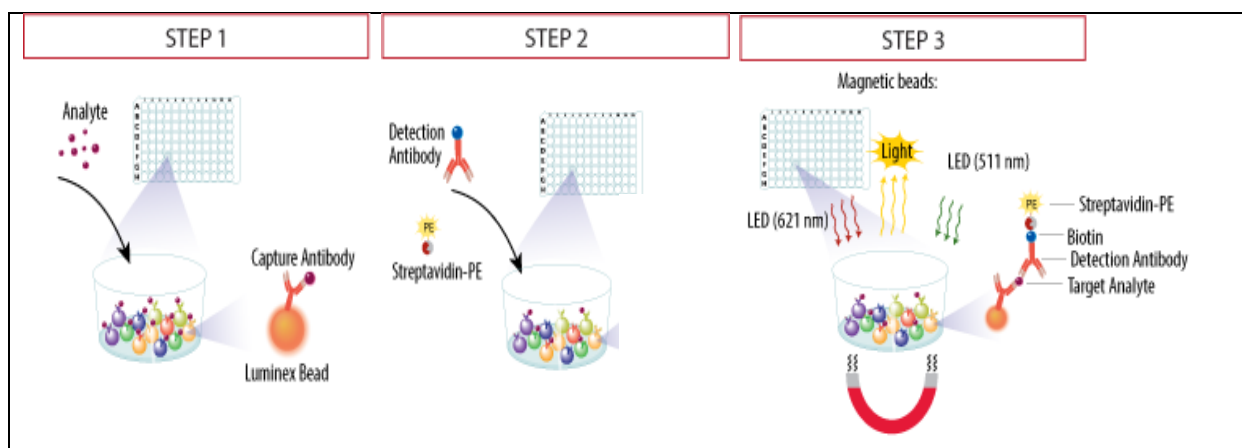


Figure 2-4 Luminex bead- based immunoassay principle.

[modified from https://www.rndsystems.com/resources/technical/luminex-bead-based-assay-principle](https://www.rndsystems.com/resources/technical/luminex-bead-based-assay-principle)

2.2.8 Data analysis

The results were exported to Windows 7 Excel 2007 spreadsheet. The statistical analyses were carried out via IBM SPSS v21 and GraphPad Prism® 6 (GraphPad Software Inc., California, USA) was used for graphical presentation. All analyses were done on data obtained until April of 2016. All data were tested for normality (using Shapiro-Wilk test) to establish the type of analysis (parametric/nonparametric). For the cross-sectional data, Mann–Whitney U test and kruskal-Wallis test followed by Dunn's post hoc test were used for non- parametric data while t- test and one-way ANOVA followed by Scheffe post hoc test were used for the normally distributed data. For the longitudinal study, the same tests were applied to determine the differences between the time points of the groups. Statistical significance was defined as a p-value of less than 0.05. For diagnostic evaluation, receiver operating characteristic (ROC) analysis was applied. The predictive power of USS was tested using chi square test. Spearman's rank correlation coefficient was used to assess the associations. Regarding the data obtained from the proteomic analysis, assessment was done using a cut- off of a logarithmic transformed fold change ratio of ≥ 2 . Data acquired from the proteome profiler arrays was tested using two methods which were the fold change cut-off of ≥ 2 and one- way ANOVA followed by Tukey HSD post hoc test. The data were plotted as fold change (\log_2 fold change ratio) and significance ($-\log_{10}$ p-value) in a volcano plot. A kruskal-Wallis test followed by Dunn's post hoc test were used to determine the differences between groups regarding all the Luminex results (screening and performance) and ELISA data. The sample size calculation for Luminex and ELISA assays was based on one-way Analysis of Variance (ANOVA) for comparing the average amount of a particular protein/cytokine present in the salivary sample between 5 different groups of participants. A study with 80% power and an effect size of 0.42 will require a total sample of 75 (15 per group) for comparing the protein levels at 5% level of significant using a two-tailed test. The power calculation was carried out using G-power version 3.1.5 software. Mean and SEM will be usually expressed in text and median and IQR will be expressed in figures unless otherwise stated.

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3 Prognostic value of clinical parameters in Sjögren's syndrome

3.1 Introduction

Other than mucosa associated lymphoid tissue lymphoma (MALT-L) development, the course of Sjögren's syndrome (SS) is generally assumed to be characterized by mild differences in disease symptoms with very slow deterioration. It is a heterogeneous multi-systemic disease diagnosed by certain criteria requiring multiple clinical and laboratory parameters (Vitali et al., 2002, Shiboski et al., 2012). The prognostic value of these parameters has been reported worldwide where different studies have investigated the long-term outcome of some of them in SS patients such as ocular disease parameters, serological parameters and clinical parameters; in Denmark (Kriegbaum et al., 1989), the Netherlands (Kruize et al., 1996) (Kruize et al., 1997), Japan (Cui et al., 1997), Poland (Chwalinska-Sadowska et al., 1998), Greece (Skopouli et al., 2000), Italy (Botsios et al., 2001), Finland (Pertovaara et al., 1999, Pertovaara et al., 2001), China (Lin et al., 2010), USA (Malladi et al., 2012) and in Hungary (Horvath et al., 2014).

Other studies have assessed autoantibody patterns and followed SS patients with lung disease (Linstow et al., 1990, Kelly et al., 1991, Davidson et al., 1999, Davidson et al., 2000, Pertovaara et al., 2004, Mandl et al., 2012). Also, the SS damage index was used to evaluate the long-term outcome in the form of growing damage in SS patients where they have reported 2 fold increase in total score damage in 10 years in 55% of patients. Unfortunately, they did not report the oral domain as it was not reliably recorded (Krylova and Isenberg, 2010). Clinical complications were addressed by another longer outcome study, demonstrating higher malignancy rate and additional autoimmune and haematological disorders over a period of 25 years (Abrol et al., 2014) .

Although the previously mentioned studies have focused more on other clinical and serological parameters, there are number of studies that have assessed the long-term course of salivary gland function (oral parameters) mainly in terms of salivary flow rates such as Jonsson et al., (1993) which was the first study to report the impact of progression of salivary gland

inflammation on salivary flow rate. Theander et al., (2005) have assessed the oral signs as part of the outcome measures and were the first to demonstrate a predictive value of laboratory tests for exocrine function in SS. Furthermore, salivary flow rate follow-up was reported by different studies (Gannot et al., 2000, Haga, 2002, Pijpe et al., 2007, Haldorsen et al., 2008). Very slow deterioration was mainly observed in studies with patients with a longer disease duration.

Salivary gland dysfunction is one of the main clinical features of SS, manifested by xerostomia with its subsequent complications and its well-known effect on the person's quality of life, though it should be noted that the complaint of xerostomia might not be correlated with actual hyposalivation. Thus, monitoring an actual oral dryness is of significant importance. Indeed, salivary gland assessments are routinely used as part of the objective diagnostic criteria (oral parameters) of SS specifically unstimulated whole flow rate (WFR) with a cut-off of 0.1 ml/min (Vitali et al., 2002). In addition, individual glandular flow rate tests may demonstrate consecutive involvement of a particular gland providing more information regarding the disease progression and variances of different stages of SS. Even though submandibular/sublingual flow rates might be useful in the early phases of SS reported by others such as Pijpe et al., (2007), parotid flow rate (PFR) might be more informative in patients with longer disease duration where parotid glands are involved especially in SS patients at risk of or already having developed lymphoma (MALT-L).

Additionally, evaluation of salivary gland changes can be done using ultrasonography, despite not being part of the diagnostic criteria, it has proven its value as a diagnostic instrument in SS in a lot of studies. A simplified ultrasound scoring system was developed (Brown, 2010). It is based on a system proposed by Hočevár et al., (2005) and it was used in this study. In spite of being not informative in early phases of SS it might be beneficial in later stages for the same reason mentioned above.

One of the methods of assessing oral dryness is The Challacombe scale or Clinical Oral Dryness Score (CODS) which uses a simple numeric system enabling a clinician to semi-quantify the severity of the xerostomia and to decide if the condition needs treatment or not. Also, it might provide a means of monitoring the progress of oral dryness over time (Challacombe et al., 2015).

Loss of salivary gland function can be noticeable in early onset of SS and progression of this functional loss seems to be even of a lower extent after a longer period of disease duration. Mostly cross-sectional and only a few longitudinal studies on the prognosis of salivary gland function in SS have been performed. This study has assessed salivary gland parameters and clinical data in SS patients with a focus on whole and parotid flow rates, CODS and USS; cross-sectional associations between each other and USS with histopathology were undertaken for the following group: disease control (DCT) dry mouth group which involves; not otherwise specified (NS), sialadenitis, nodal osteoarthritis & xerostomia (SNOX) and drug induced hyposalivation (DIH). For SS groups, they were SS with or without other autoimmune disease, SS at risk of developing MALT-L and those who developed MALT-L. Moreover those 4 oral parameters were followed in a cohort of patients over 5 and 10 years and their progression was noted. Description of the classical predictors of lymphoma in SS patients was done as well as detailed records of MALT-L patients.

3.2 Aims and objectives

Salivary gland assessments are commonly used in diagnosing Sjögren's syndrome (SS) patients and it is of great significance to evaluate their ability to stratify different subgroups of SS and to consider their long-term outcome. Our hypothesis was that measurements of salivary gland involvement; whole mouth and parotid flow rates, clinical oral dryness and ultrasound scores can differentiate between SS subgroups and other dry mouth patients. The secondary hypothesis was that SS does show on-going salivary function loss over time.

To enable better understanding of the disease activity and progress the following aims were pursued:

1. Unravel whether the measurements of salivary gland involvement can help classify SS patients into the different subgroups (SS, SS at risk of developing MALT-L and SS with MALT-L) and whether they can differentiate them from other dry mouth patients (NS, SNOX and DIH).
2. Establish whether there is an overall association between the above-mentioned parameters.
3. Correlate the ultrasound scores with diagnostic focus scores as well as measuring their levels of agreement.
4. Evaluate the diagnostic accuracy of the ultrasound scores.

Also in this study, a cohort of patients was followed- up and the following specific aim was undertaken:

5. Complete a longitudinal study on dry mouth patients at 5 and 10-year time-points to investigate deterioration in salivary function over time.

3.3 Materials and methods

3.3.1 Study group

Patients with symptomatic dry mouth attending Guy's and St. Thomas's Hospital Oral Medicine department (GSTT NHS Foundation Trust) were included. Patients who fulfilled the American-European Consensus Group (AECG) criteria (Vitali et al., 2002) were included as SS patients while others were considered as disease control subjects according to their relevant findings; drug induced hyposalivation (DIH), Sialadenitis, nodal osteoarthritis and xerostomia (SNOX) and Not otherwise specified (NS). Dry mouth patients were selected from the patients' records where few patients were considered as newly diagnosed patients. Further subgrouping of SS patients involved presence of another autoimmune disease (e.g. rheumatoid arthritis, systemic lupus erythematosus, scleroderma and primary biliary cirrhosis). Patients with lymphoma (MALT-L) were also recorded whenever found. SS patients were considered at risk of developing MALT-L if they had at least three of any of the commonly reported risk factors (Nocturne and Mariette, 2015), the markers of severe SS (e.g., parotid enlargement, hypocomplementemia, palpable purpura, cryoglobulinemia, high focus score, germinal centre in their biopsy and previous lymphoma) are associated with a more pronounced risk of NHL, hence they were subgrouped as high risk patients while the low risk group were associated with other generalised inflammatory factors such as raised β_2 microglobulin levels, lymphopenia and hypergammaglobulinemia. In addition, higher ultrasound scores with the presence of the hypo-echoic foci were also considered as one of the predictive factors.

3.3.2 Data collection

Electronic patient records (EPR) of 366 patients during the years of 2005 to early 2016 were initially examined; medical notes of patients were used to follow subjects who attended for earlier durations. All patients had been or are currently being followed at clinics. Clinical parameters; whole and parotid flow rates, ultrasound and dry mouth scores (USS and CODS) were recorded for all the patients when present. Date from first visit and last visits were logged in order to subgroup the patients into their relevant categories; follow-up of 3 years or less was considered for the cross-sectional study while 10 ± 2 and 5 ± 1 year follow-up for the longitudinal study, also patients' data at baseline was included in the cross-sectional analysis. Furthermore, patients who were due for a 5 or 10-year follow-up visit, who appeared to satisfy the MALT-L risk diagnosis or with confirmed MALT-L were invited to clinics for this study; altogether, 129 patients consented to a new clinical and laboratory investigation (samples were used for the subsequent chapters 4 and 5). These investigations included whole and parotid flow rates recordings while measurement performed by (AJ) and some of the clinical staff so as for CODS and USS was performed by specialist radiologist. Patients with full records at baseline and follow-up were considered for the longitudinal analysis while those with one complete set of data at one time were for cross-sectional analysis; those with incomplete data of the 4 parameters at a single time or still with a provisional diagnosis were excluded. Cross-sectional comparisons at baseline were done between the different groups of patients before proceeding with the long-term comparisons.

3.3.3 Data analyses

For the cross- sectional analysis, comparison between 2 groups (disease controls vs. overall SS groups) was done via Mann–Whitney U tests then Kruskal-Wallis test followed by Dunn's post- hoc tests to determine the differences between the multiple groups (SS subdivisions) for parameters that were not normally distributed (PFR, WFR and USS). CORDS were normally distributed thus one- way ANOVA followed by Scheffe post hoc test were used for multiple comparisons and independent sample t- tests for comparing 2 groups (disease controls vs. all SS groups). Spearman's rank correlation coefficient (Spearman's rho) was used to determine the association of these 4 parameters. Discriminant validity was assessed via Receiver Operator Characteristic (ROC) curves to determine the ability of USS to differentiate between SS subgroups and non- SS group. Negative and positive predictive values as well as odds ratios were calculated from contingency tables. Cohen's kappa coefficient was used to measure the level of agreement between biopsy focus scores and USS while Spearman's rank correlation coefficient (Spearman's rho) was used to assess their relationship. For the longitudinal study, the same tests were applied to determine the differences between the time points of each parameter of the groups. The median and quartiles were used as estimates of central tendency and dispersion of non -parametric data while means and standard error of mean SEM were used to demonstrate the parametric data.

3.4 Results

3.4.1 Cross- sectional study (Study group)

Out of 366 patients, 122 were excluded due to insufficient data. The remaining 244 patients were included in a cross- sectional analysis and of them 80 patients were followed over a 5- year period while only 16 could be followed over a 10- year period with complete records of all 4 parameters. The remaining patients (n=148) were either considered recently diagnosed patients, had ≤ 3 years follow up (n= 117) or were patients with only one complete set of records at a time point (baseline) but not at follow-up (n=31). The 244 patients consisted of non- SS patients (n=70) (25 DIH, 20 NS, and 25 SNOX) and SS patients (n=174; 119 without and 55 with another autoimmune disease). Thirty of the SS patients were at risk of MALT-L and 26 had MALT-L. A summary of the different diagnostic groups for the cross- sectional study is shown in **table 3-1**. A schematic work flow of data collection is shown in **figure 3-1**.

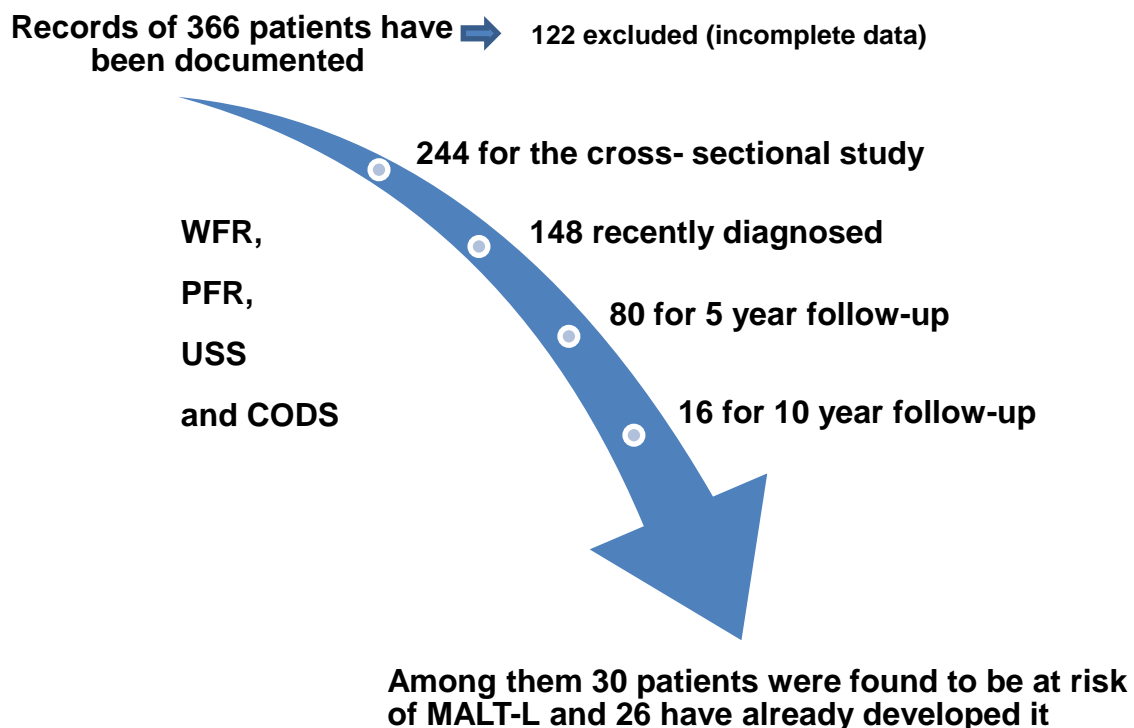


Figure 3-1 Study identification and selection flow chart.

WFR; unstimulated whole flow rate, PFR; stimulated parotid flow rate, USS; ultrasound score, CODS; clinical oral dryness score.

Table 3-1 Demographics and clinical characteristics of the SS patients and disease control subjects included in the cross-sectional analysis (n=244)

Diagnosis	Disease controls (n=70)			SS groups (n=174)			
	SNOX	DIH	NS	SS	SS at risk	SS/ MALT-L	Overall SS
Number (%)	25 (36)	25 (36)	20 (28)	118 (68)	30 (17)	26 (15)	174 (100)
				SS			
				SS+			
				80 (68)	22 (73)	17 (65)	119 (68)
				38 (32)	8 (27)	9 (35)	55 (32)
Age, mean (SEM)	63.1 (1.9)	59 (2)	51 (3.4)	52 (1.1)	48.1 (2)	51.7 (2.8)	51.3 (0.9)
Gender, n (%)	F	23 (92)	21 (84)	18 (90)	109 (92)	30 (100)	26 (100)
	M	2 (8)	4 (16)	2 (10)	9 (8)	0	0
Anti SSA/SSB positive, n (%)	0	0	0	78 (67) 34 only SSA	28 (93) 3 only SSA	23 (88) 1 only SSA	129 (74)
ANA positive, n (%)	1 (4)	4 (16)	3 (15)	64 (50)	24 (80)	20 (77)	108 (64)
Rf positive, n (%)	3 (12)	5 (20)	4 (20)	82 (69)	29(97)	24 (92)	135 (78)
Biopsy positive, n (%)	0	0	0	105 (89)	29 (97)	25 (96)	159 (95)
	[non-specific]	[normal]	[normal]	12 [non-specific]	1 [non-specific]	1 [non-specific]	
	6 ND	11 ND	4 ND	1 [normal]			

SNOX; sialadenitis, nodal osteoarthritis & xerostomia, DIH; drug induced hyposalivation, NS; not otherwise specified, SS; Sjogren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, SS at risk; SS at risk of developing MALT-L, SS+; SS with other autoimmune disease, RF; Rheumatoid factor biopsy positive; positive for focal sialadenitis, [non- specific sialadenitis], ND; not done.

3.4.1.1 SNOX as part of the disease control group

Of the 70 disease control patients 7 were followed over 5 year while one only was followed over 10 years, 25 patients (36%) appear to fulfil the description of SNOX (17 in the recently diagnosed group and 7 in the 5- year follow up while 1 is in the 10- year); none were on xerostomic drugs and 19 (76%) of them had a diagnosis of non- specific sialadenitis according to their biopsy results while the other 6 patients have not had biopsies yet but they had the rest of the features of osteoarthritis, xerostomia, negative anti SSA/SSB. Three (12%) were positive for Rheumatoid factor. There was a female predominance (23 vs. 2) and they were of an older age group (62 vs. 51). Although they complained of xerostomia but their WFR (0.14 ± 0.03 ml/min; mean \pm SEM) were not below the accepted cut-off nor were their parotid flow rates (0.3 ± 0.08 ml/min). Their CORDS were 4 ± 0.5 . Only 5 patients had an average USS of 3 and 4 patients with an average of 4 while the remaining patients had scores of zero. These patients are clearly distinguishable from SS patients and almost half of them were chosen for the laboratory analysis in the next chapters.

3.4.1.2 SS sub group

Of the 118, 45 were followed over 5 years and 6 over 10 years. SS patients included in this study, 80 were diagnosed as having SS and 38 as having SS with other autoimmune disease. Sixty-seven % were ENA positive while 50% were ANA positive, 69 % RF positive and 89% biopsy positive having a focus score of one or more while 12 patients had evidence of non-specific sialadenitis and one patient showed normal gland histology. Forty-three parotid saliva samples (and whole if present) were collected and half of them were used for the following chapters. Interestingly some parotid saliva samples (n=7) were mucus like secretions of patients who had longer SS duration (3 in the 10-year follow –up group, 3 in the 5- year group while one was in the recently diagnosed group).

3.4.1.3 MALT-L risk sub group

From the cohort of 30 patients at risk of developing MALT-L, 13 were recently diagnosed (≤ 3 years or new patients), 13 patients were in the 5-year follow-up category and 4 patients were in the 10-year category and all of them are still current patients. Demographics and clinical characteristics for each group are shown in tables 3-1, -2 and -3. Further subgrouping has revealed that patients that were classified as low risk of developing lymphoma ($n=7$) were mostly in the recently diagnosed group. 5/13 patients (38%) vs. 2/13 patients (15%) in the 5-year category and none in the 10 year. This group had at least three of the following; consistently raised $\beta 2$ microglobulin levels, lymphopenia and hypergammaglobulinemia or raised ultrasound scores. Twenty-three patients were classified as high risk; 8/13 patients (62%) of the newly diagnosed group, 11/13 (85%) of the 5-year group and all 4 (100%) of the 10-year group. Of the 23, 14 patients (61%) had raised $\beta 2$ microglobulin levels, 15 (65%) had either consistent or recurrent parotid swelling. Fifteen (65%) had hypergammaglobulinemia, 15 (65%) with high focus score of 4 and above and germinal centres were present in 4 of them. Fourteen (61%) had lymphopenia, 12 (52%) had high ultra sound scores with hypo-echoic foci (scores of 8 and 9) while another 10 (43%) patients had scores of 5-7 with the presence of the foci, 10 (43%) with hypocomplementemia, 5 (22%) with lymphadenopathy, 4 (17%) with vasculitis, 2 (10%) with cryoglobulinemia, 2 (10%) with polyclonal expansion and 1 (4%) with glomerulonephritis. One patient had a chest lymphoma and another 3 had gastric, Hodgkin's and plasmacytic lymphoma respectively. One patient had a confirmation of MALT-L during the period of follow-up. In addition, other types of cancer were noted in some of the patients (e.g., melanoma, thymoma, ovarian and breast cancer). Twenty-seven parotid saliva samples (and whole saliva if present) were collected while 3 were not collected and 18 samples were used for the subsequent studies (chapters 4 and 5) while 9 samples were not used; mucus like secretions (plugs) which were difficult to analyse. Fourteen (52%) patients were either treated with steroids or hydroxychloroquine by time of sample collection.

3.4.1.4 MALT-L sub group

From the cohort of 26 patients that developed MALT-L, 6 were recently diagnosed (≤ 3 years or new patients), 15 patients were in the 5- year follow- up category and 5 patients were in the 10-year category. Demographics and clinical characteristics for each group are found in tables 3-1, 3-2 and 3-3. All patients are still currently seen at clinics while one patient died in the course of the disease due to interstitial lung disease. Six patients (23%) had bilateral parotid involvement. Ten (38%) were in left parotids and one of them had mediastinal MALT-L as well, while the right side was noted in 5 patients (19%) and one of them had gastric MALT-L as well. Left submandibular gland involvement was found in one patient (4%) and another 2 (8%) were confirmed via buccal and labial biopsy respectively. Interestingly one patient had nasal involvement while another was at risk group but confirmed upon biopsy of the parotid. Four of them (15%) had monoclonal expansion. 17 patients (65%) were SS patients only while the rest 9 (35%) were SS with other autoimmune disease. One patient had a recurrent lymphoma following partial paridectomy 4 years later.

An attempt was made to collect 24 parotid saliva samples (and whole mouth saliva if present) Two patients did not produce any saliva (parotid or whole) while six samples were collected with sufficient volume but the volumes of the remaining samples were too low to calculate a flow rate (used only for subsequent chapters 4 and 5). Eight of these parotid saliva samples were mucus (plug) like secretions. Treatment was commenced following MALT-L diagnosis and it was surgical excision (partial or full) (mostly done in the past) for 13/26 patients (50%) followed by chemotherapy or radiotherapy in some cases. For the rest of the other 13 patients a watch and wait policy (observation) was adopted for 6 (23%) patients. Three patients (13%) had radiotherapy (4 Gy in two fractions) (low intensity radiation) while three had chemotherapy (10 mg chlorambucil cycles). One patient had rituximab treatment.

3.4.1.5 Clinical parameters outcome

3.4.1.5.1 WFR

The overall median (Q1-Q3) unstimulated whole flow rate for the SS group 0.04 (0- 0.16) was significantly reduced when compared to the disease control group 0.12 (0.1- 0.24) ($p < 0.0001$). Subgrouping of SS has revealed reduction of flow rates of the MALT-L risk group 0.01 (0- 0.1) and MALT-L group 0.002 (0- 0.06) when compared to control group ($p < 0.0001$ for both) as well as when they were compared to SS subgroup 0.08 (0- 0.24) ($p = 0.039$ and 0.049 respectively). No differences were detected between the SS subgroup and disease control group (**figure 3-2**).

3.4.1.5.2 PFR

Median (Q1-Q3) stimulated parotid flow rate of the overall SS group 0.14 (0- 0.3) was statistically lower than the disease control group 0.28 (0.16- 0.44) ($p < 0.0001$) while when it was subgrouped; at risk 0.01 (0- 0.25) and MALT- L 0 (0-0.13) groups were reduced when compared with disease control group ($p = 0.005$ and < 0.0001 correspondingly) whereas only MALT-L group was reduced compared to the SS subgroup 0.22 (0- 0.33) ($p = 0.022$). SS subgroup was not reduced in comparison to the disease control group (**figure 3-3**).

3.4.1.5.3 USS

Median (Q1-Q3) ultrasound score of the overall SS group 5 (4-7) was significantly higher than the disease control group 0 (0-0) ($p < 0.0001$) while subgrouping SS has shown an increased means of all the SS subgroups; SS 5 (3-5), at risk 6.5 (5-8) and MALT-L 7.5 (6-9) when compared to disease control ($p < 0.0001$ in all). In addition, both at risk and MALT-L were increased when compared to SS subgroup ($p = 0.001$ and $p < 0.0001$ respectively) (**figure 3-4**).

3.4.1.5.4 CODS

Median (Q1-Q3) dry mouth score of the overall SS group 5 (3-7) was higher than the disease control group 3 (1-5) ($p < 0.0001$) as were the subgroups; SS 5 (2-7) at risk 5 (3-7) and MALT-L 7.5 (5-8) ($p=0.013$, 0.005 and <0.0001 respectively). Amongst the subgroups, MALT-L was higher than SS subgroup ($p=0.006$) (**figure 3-5**).

All parameters (WFR, PFR, USS and CODS) showed no differences between SS with and without another autoimmune disease. In addition, no differences were detected between patients at high and low risk of developing MALT-L except for USS where patients at high risk had an increased median score (Q1-Q3) of 7 (6-9) compared to the low risk group 5 (3-6) ($p=0.029$) (**figure 3-6**).

3.4.1.6 Associations

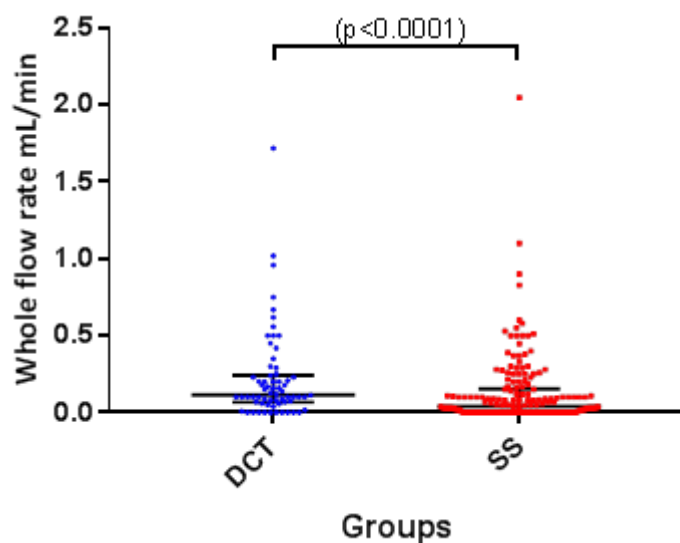
All the SS disease measures (clinical parameters) correlated well with each other in cross-sectional analyses at baseline ($n=244$), notably whole flow rate with parotid flow rate and dry mouth score. Ultrasound scores showed a reasonable correlation with all the measures as shown in **table 3-2**.

Table 3-2 Spearman correlation of the clinical parameters of the cross- sectional study

Spearman's rho				
	WFR	PFR	USS	CODS
WFR	1			
PFR	.604**	1		
USS	-.39**	-.37**	1	
CODS	-.59**	-.503**	.39**	1

WFR; whole flow rate, PFR; parotid flow rate, USS; ultrasound score, CODS; clinical oral dryness score. ** $p < 0.0001$. Correlation is significant at the 0.01 level (2-tailed)

(A)



(B)

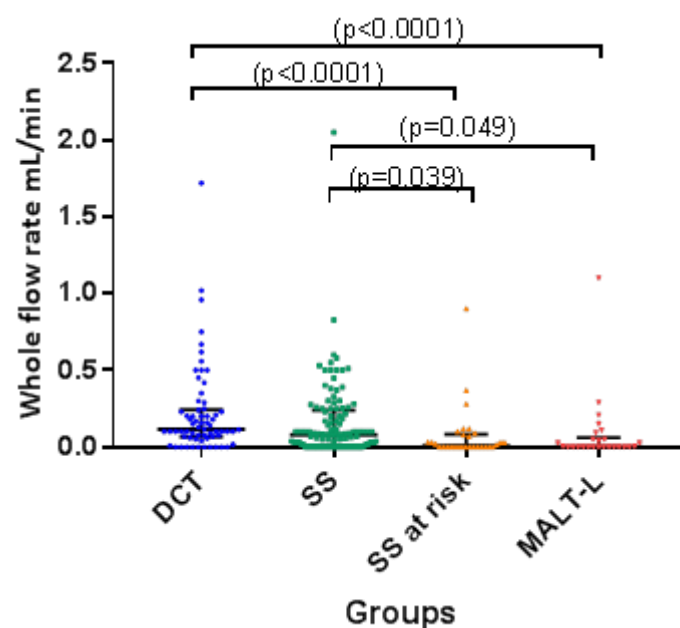
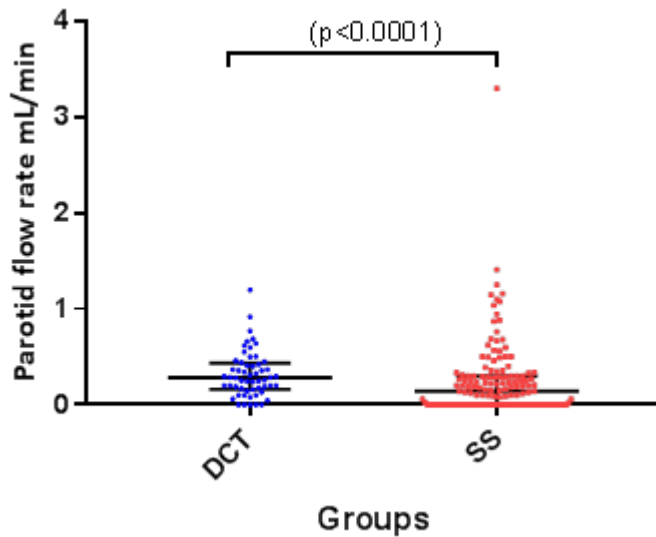


Figure 3-2 Whole mouth saliva flow rates of different groups (cross-sectional) (A) SS groups are combined (B) SS subgroups. n= (244)

DCT; disease controls which are not otherwise specified (NS), sialadenitis, nodal osteoarthritis & xerostomia (SNOX) and drug induced hyposalivation (DIH), SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, SS at risk; SS at risk of MALT-L. Data are reported as median \pm (IQR) and expressed as mL/min.

(A) Mann-Whitney U test (B) Kruskal-Wallis test followed by Dunn's post hoc test.

(A)



(B)

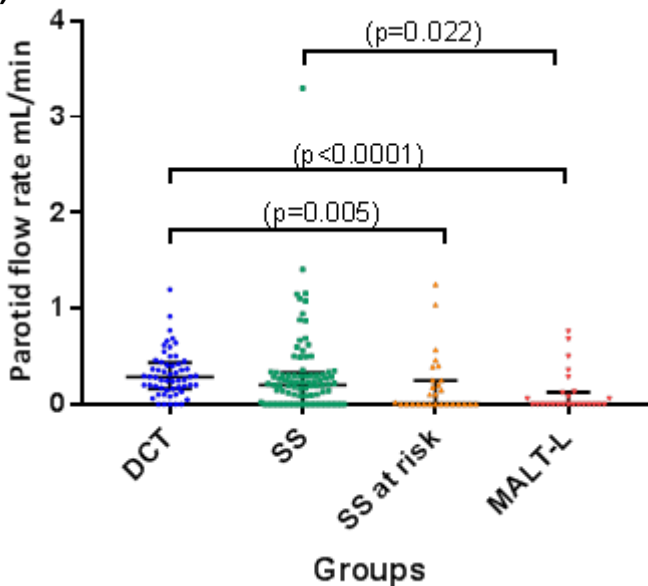


Figure 3-3 Parotid saliva flow rates of different groups (cross- sectional) (A) SS groups are combined (B) SS subgroups. n= (244)

DCT; disease controls which are not otherwise specified (NS), sialadenitis, nodal osteoarthritis & xerostomia (SNOX) and drug induced hyposalivation (DIH), SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, SS at risk; SS at risk of MALT-L. Data are reported as median \pm (IQR) and expressed as mL/min.

(A) Mann–Whitney U test (B) Kruskal-Wallis test followed by Dunn's post hoc test.

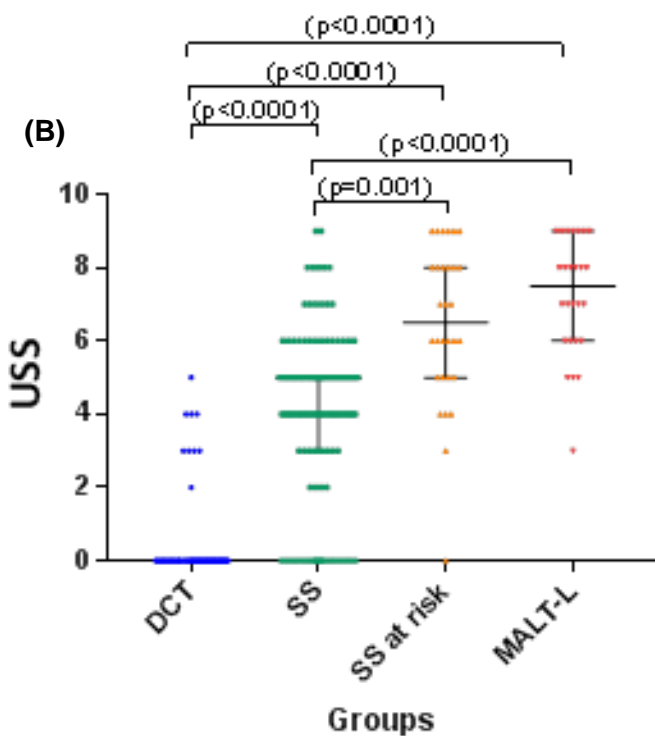
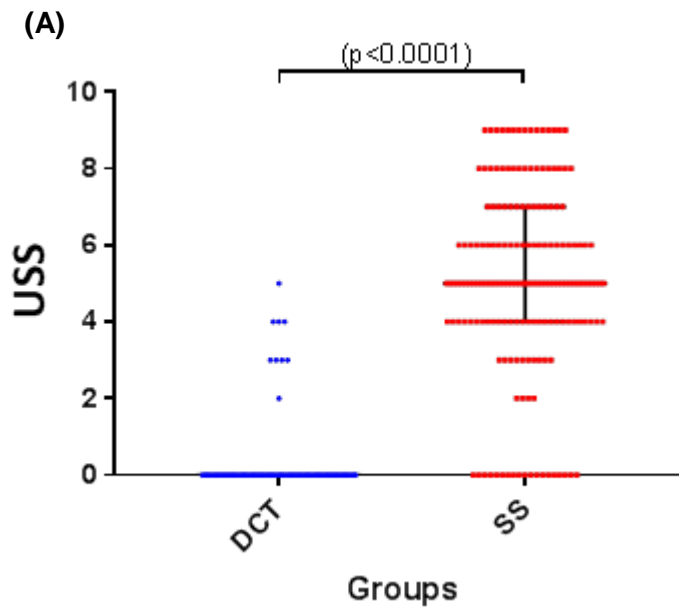


Figure 3-4 Ultrasound scores of different groups (cross- sectional) (A) SS groups are combined (B) SS subgroups. n= (244)

DCT; disease controls which are not otherwise specified (NS), sialadenitis, nodal osteoarthritis & xerostomia (SNOX) and drug induced hyposalivation (DIH), SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, SS at risk; SS at risk of MALT-L. Data are reported as median \pm (IQR).

(A) Mann–Whitney U test (B) Kruskal-Wallis test followed by Dunn's post hoc test.

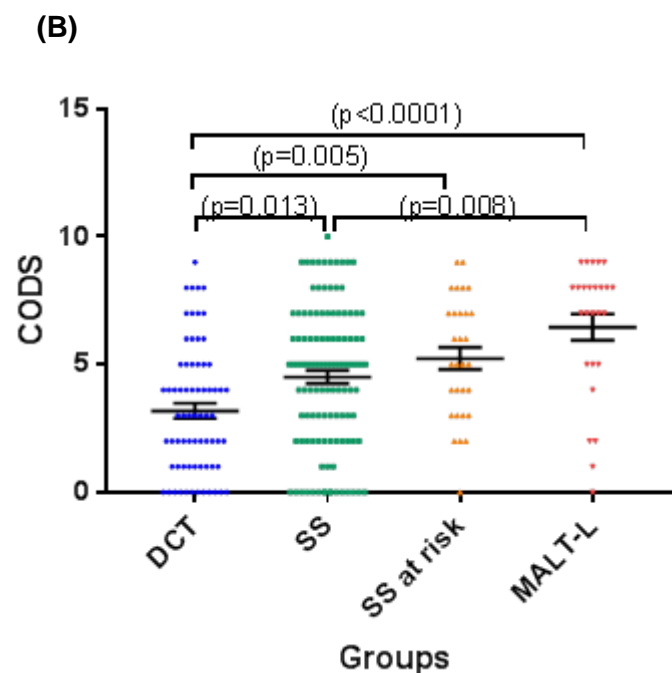
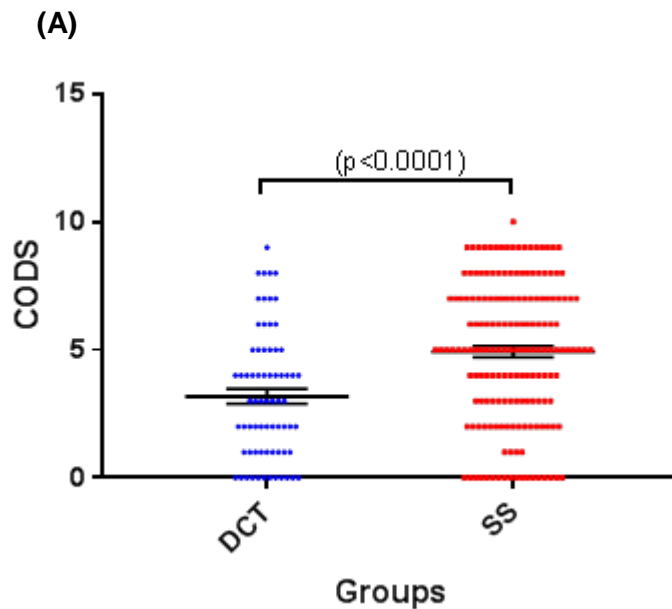


Figure 3-5 Clinical oral dryness score (CODS) of different groups (cross-sectional) (A) SS groups are combined (B) SS subgroups. n= (244)

DCT; disease controls which are not otherwise specified (NS), sialadenitis, nodal osteoarthritis & xerostomia (SNOX) and drug induced hyposalivation (DIH), SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, SS at risk; SS at risk of MALT-L. Data are reported as mean \pm (SEM).

(A)Independent t- test (B) One- way ANOVA followed by Scheffe post hoc test.

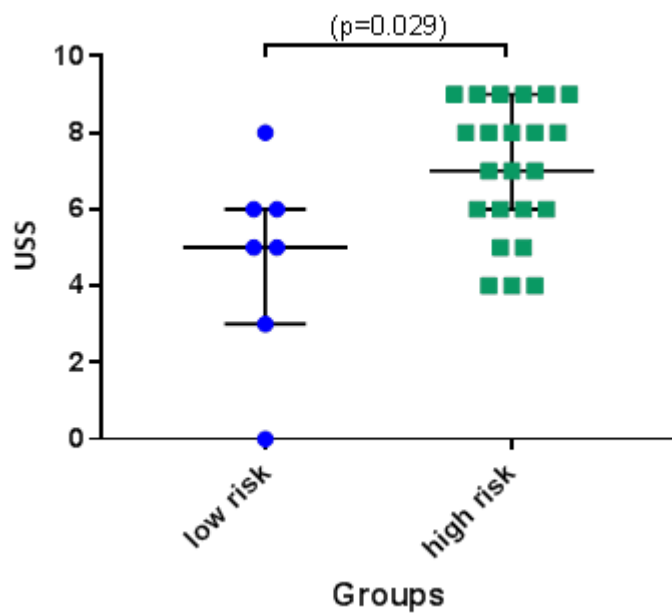


Figure 3-6 Ultrasound score of SS patients at low and high risk of developing mucosa associated lymphoid tissue lymphoma (MALT-L)

Classified as igh risk patients (n=23) and low risk patients (n=7). Data are reported as median \pm (IQR). (Mann–Whitney U test).

3.4.1.7 Diagnostic accuracy of USS

3.4.1.7.1 Receiver operator characteristic (ROC) curve

From the optimal cut off points that were computed based on optimum separation via ROC curves, USS ≥ 4 was selected and it displayed 81.03% sensitivity (95% CI: 74.41- 86.57) and 94.29% specificity (95% CI: 86.01- 98.42). Area under the curve (AUC) was 0.92 (95%CI; 0.89 to 0.96) and $p < 0.0001$ with a likelihood ratio of 7.543 (**figure 3-7**). Further points based on the maximum sum of sensitivity and specificity were selected and ROC curves were tested further on the subgroups of SS summarised in **table 3-3**.

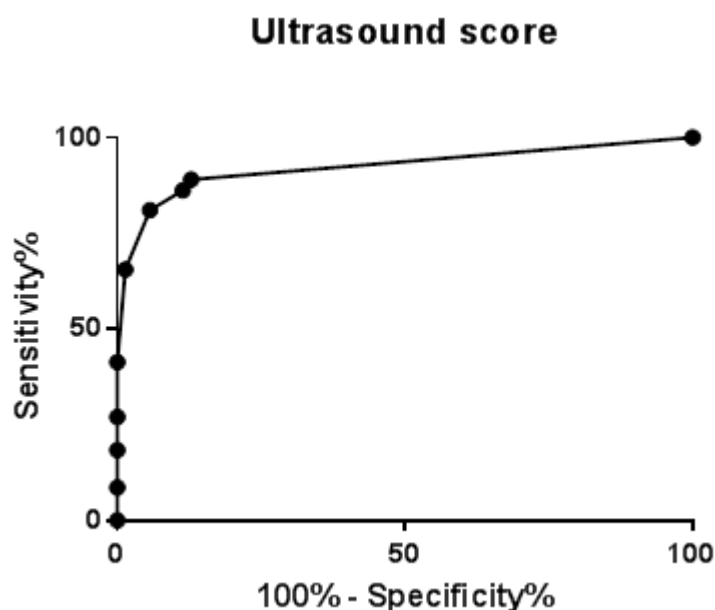


Figure 3-7 ROC curve of ultrasound score in identifying patients with SS
Overall SS (n=174) and non-SS (disease controls) (n=70) total (n=244).

Table 3-3 ROC curve of ultrasound score in identifying patients with SS subgroups

Roc curve					
Ultrasound	Cut- off score	Sensitivity (%) (95%CI)	Specificity (%) (95%CI)	AUC	P value
DCT vs. SS	4	74.58 (65.74- 82.14)	94.29 (86.01- 98.42)	0.89	<0.0001
DCT vs. SS at risk	5	83.33 (65.28- 94.36)	98.57 (92.3- 99.96)	0.97	<0.0001
DCT vs. SS/MALT-L	5	84 (65.35- 93.6)	98.57 (92.3- 99.96)	0.99	<0.0001
SS vs. SS at risk	6	70 (50.6- 85.27)	74.58 (65.74- 82.14)	0.76	<0.0001
SS vs. SS/MALT-L	6	84 (63.92- 95.46)	74.58 (65.74- 82.14)	0.86	<0.0001
At risk vs. SS/MALT-L	7	68 (46.5- 85.05)	50 (31.3- 68.7)	0.6	NS

Optimal cut- off scores of ultrasound scores, DCT; disease controls, SS; Sjögren's syndrome, SS/MALT-L; SS with mucosa associated lymphoid tissue lymphoma, SS at risk; at risk of MALT-L, AUC; area under the curve.

3.4.1.7.2 Positive and negative predictive values (PPV and NPV)

The above selected cut- offs were used to generate a 2X2 contingency tables in order to estimate the positive predictive value (PPV), negative predictive value (NPV) and odds ratio where chi square tests were used. An example of the comparison between the disease controls and overall SS groups is provided whilst the rest are summarised in **table 3-4**.

X² analysis of 244 patients			
Data analysed	Overall Sjögren's syndrome	Disease controls	Total
USS + (> OR =4)	(True positives) 141	(False positives) 4	145
USS - (<4)	(False negatives) 33	(True negatives) 66	99
Total	174	70	244
(Chi square=10.84; p<0.0001)		PPV=97.24% (95%CI; 93.12- 98.92)	
Odds ratio= 70.5% (95%CI; 24.16- 187.3)		NPV=66.67% (95%CI; 56.91- 75.18)	

Table 3-4 Summary of chi square results on SS subgroups

SS subgroups					
Ultrasound	Cut-off score	PPV (%) (95%CI)	NPV (%) (95%CI)	Odds ratio (%)	P value
DCT vs. SS	4	95.6 (89.24- 98.28)	68.04 (58.23- 76.48)	46.31	<0.0001
DCT vs. SS at risk	5	96.15 (81.11- 99.86)	93.24 (85.14- 97.08)	345	<0.0001
DCT vs. SS/MALT-L	5	95.45 (78.2- 99.77)	94.52 (86.74- 97.85)	362	<0.0001
SS vs. SS at risk	6	40.38 (28.16- 53.93)	90.63 (83.13- 94.99)	6.54	<0.0001
SS vs. SS/MALT-L	6	40.38 (28.16- 53.93)	95.6 (89.24- 98.28)	14.73	<0.0001
At risk vs. SS/MALT-L	7	46.88 (30.87-63.55)	34.78 (18.8-55.11)	0.47	NS

DCT; disease controls, SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, at risk; at risk of MALT-L, NPV; negative predictive value, PPV; positive predictive value, NS; not significant.

3.4.1.7.3 Association with focus scores

The pathology results of labial gland biopsies from a total of 62/174 SS patients were recorded; there were 43 patients in the SS subgroup with a mean score \pm SEM of 3.7 ± 0.04 , 11 MALT-L risk patients with a mean score of 5.2 ± 0.6 and 8 MALT-L patients with a mean score of 6.6 ± 0.6 . The scores for 57/70 disease control patients were scored 0 as their pathology results were either normal gland or non-specific sialadenitis. Biopsies were not done for the rest of controls while the remaining SS patients were done but not scored. A significant positive correlation was noted between the USS and focus score of the overall group (spearman $r=0.7$, $n=119$, $p < 0.0001$) and a considerable correlation in the SS groups (spearman $r=0.3$, $n=62$, $p < 0.0001$).

3.4.1.7.4 Measurement of agreement

An ultrasound score of ≥ 4 was considered positive for SS patients and was used to sort them accordingly to positive and negative disease groups. The focus score of ≥ 1 per 4 mm^2 was considered positive for SS and patients were sorted as well. Cohen's kappa (κ) was performed in order to determine if there was agreement between the two methods, there was a good agreement, $\kappa = 0.748 \pm (\text{SEM}) 0.061$, $p < .0001$. The overall agreement was calculated as 87.3% and the USS results had a PPV of 88.1% and a NPV of 89.6% of the biopsy results; out of the 60 patients with negative biopsy results, 8 were positive for ultrasound while out of the 59 patients with positive biopsy results, 7 were negative for ultrasound.

3.4.2 Longitudinal study (Study group)

Eighty patients were followed over 5 years (7 non- SS patients and 73 SS patients; 45 SS, 13 SS at risk and 15 already developed MALT-L) while only 16 SS patients were followed for 10 years (6 SS, 4 at risk and 5 with MALT-L) and only one of them was a SNOX patient. Detailed demographics and clinical characteristics are found in **tables 3-6 and 3-7**.

3.4.2.1 Five-year follow- up

The mean follow-up period (mean \pm SEM) was 5.5 ± 1.6 years with a range from 4 to 7 years for the 80 patients. During the follow-up period, WFR levels were found to not change between all of the groups of patients (DCT vs. overall SS) ($p=0.33$) and (DCT vs. SS subgroups) ($p=0.5$), as well as for the PFR values (DCT vs. overall SS) ($p=0.87$) and (DCT vs. SS subgroups) ($p=0.8$).

No statistically significant changes were seen between the mean values of dry mouth scores (CODS) at baseline and follow-up between all the tested groups (DCT vs. overall SS) ($p=0.54$) and (DCT vs. SS subgroups) ($p=0.57$).

Mean values of USS did not change at baseline and follow- up period between all of the tested groups (DCT vs. overall SS) (0.78) and (DCT vs. SS subgroups) ($p=0.5$). We observed no differences between SS patients with and without other autoimmune disease, or between MALT-L risk patients (low vs. high) regarding all the parameters (Mann–Whitney U test or Independent t-test).

3.4.2.2 Ten-year follow- up

Sixteen patients were included with a mean follow-up period (mean \pm SEM) of 11.3 \pm 2.4 years and a range from 8 to 13 years. Only one disease control patient (SNOX) was included. WFR of the overall SS remained at their already very low level starting with a very low reading and continued having zero reading at later timepoints afterwards. PFR of the overall SS remained the same during the follow- ups. While USS of the overall SS showed only a slight non-significant deterioration during the second follow - up. CORDS for the overall SS groups was increased insignificantly during the second follow-up and remained stable afterwards. None of the above-mentioned parameters showed a significant change (**table 3-5**).

Table 3-5 SPSS analysis of the clinical parameters in the 10- year follow- up analysis

Hypothesis Test Summary				
	Null Hypothesis	Test	Sig.	Decision
1	The distribution of DMS12_DIFF is the same across categories of Group.	Independent-Samples Kruskal-Wallis Test	.460	Retain the null hypothesis.
2	The distribution of USS12_DIFF is the same across categories of Group.	Independent-Samples Kruskal-Wallis Test	.231	Retain the null hypothesis.
3	The distribution of WFR12_DIFF is the same across categories of Group.	Independent-Samples Kruskal-Wallis Test	.149	Retain the null hypothesis.
4	The distribution of PFR12_DIFF is the same across categories of Group.	Independent-Samples Kruskal-Wallis Test	.163	Retain the null hypothesis.
5	The distribution of DMS23_DIFF is the same across categories of Group.	Independent-Samples Kruskal-Wallis Test	.181	Retain the null hypothesis.
6	The distribution of USS23_DIFF is the same across categories of Group.	Independent-Samples Kruskal-Wallis Test	.929	Retain the null hypothesis.
7	The distribution of WFR23_DIFF is the same across categories of Group.	Independent-Samples Kruskal-Wallis Test	.191	Retain the null hypothesis.
8	The distribution of PFR23_DIFF is the same across categories of Group.	Independent-Samples Kruskal-Wallis Test	.967	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

WFR; whole flow rate, PFR; parotid flow rate, USS; ultrasound score, DMS=CORDS; clinical oral dryness score. The first four groups (comparisons) were done when SS was combined while the second 4 groups were done when SS was subgrouped.

Table 3-6 Demographics and clinical characteristics of the SS patients and disease control subjects included in the 5-year longitudinal analysis (n=80)

		Disease controls (n=7)		SS patients (n=73)			
Diagnosis		SNOX		Overall SS (n=73)	SS (n=45)	SS at risk (n=13)	SS/ MALT-L (n=15)
Number (%)		6 SNOX (86)	SS	44 (60)	28 (62)	10 (77)	6 (60)
		1 DIH (14)	SS+	29 (40)	17 (38)	3(23)	9 (40)
Age, mean (SEM)		58.3 (4.4)		51.4 (1.3)	50.7 (1.6)	51.2 (2.6)	52.9 (3.9)
Gender, n (%)	F	23 (92)		68 (93)	40 (89)	13 (100)	15 (100)
	M	2 (8)		5 (7)	5 (11)	0	0
		Median (Q1-Q3)		Median (Q1-Q3)	Median (Q1-Q3)	Median (Q1-Q3)	Median (Q1-Q3)
^ WFR at baseline		0.2 (0.09- 0.42)		0.03 (0- 0.11)	0.07 (0- 0.16)	0 (0- 0.1)	0 (0- 0.03)
WFR at follow-up		0.1 (0.05- 0.15)		0 (0- 0.14)	0.05 (0- 0.16)	0 (0)	0 (0)
^ PFR at baseline		0.4 (0.34- 0.5)		0.12 (0- 0.3)	0.2 (0- 0.5)	0 (0- 0.3)	0 (0- 0.1)
PFR at follow-up		0.4 (0.15- 0.6)		0.1 (0- 0.3)	0.2 (0- 0.5)	0 (0- 0.2)	0 (0- 0.13)
		Mean (SEM)		Mean (SEM)	Mean (SEM)	Mean (SEM)	Mean (SEM)
USS at baseline		0.7 (0.7)		5 (3.5)	4.6(0.3)	6.6 (0.5)	7.5(0.4)
USS at follow-up		0.6 (0.6)		5 (1)	4.8 (0.2)	6.3 (0.4)	6.9(0.5)
CODS at baseline		3 (1.2)		5.4 (0.3)	4.7 (0.4)	5.9 (0.7)	7.2(0.5)
CODS at follow-up		2.6 (1.1)		6 (0.3)	5.5 (0.4)	6.7 (0.5)	7.4 (0.5)

SNOX; sialadenitis, nodal osteoarthritis & xerostomia, DIH; drug induced hyposalivation, SS; Sjogren's syndrome, +; with another autoimmune disease, MALT-L; mucosa associated lymphoid tissue lymphoma. WFR; whole flow rate, PFR; parotid flow rate, USS ultrasound score, DMS; dry mouth score

[^] (ml/min), Q1; first quartile, Q3; third quartile. Data are reported as median± (IQR) or mean± (SEM).

Table 3-7 Demographics and clinical characteristics of the SS patients and one disease control patient included in the 10-year longitudinal analysis (n=16)

Diagnosis	Disease controls (n=1)		SS patients (n=15)			
	SNOX		Overall SS (n=15)	SS (n=6)	SS at risk (n=4)	SS/ MALT-L (n=5)
Number (%)	1	SS	12 (80)	5(83)	4 (100)	3 (60)
		SS+	3 (20)	1(17)	0	2 (40)
Age, mean (SEM)						
At first visit	64		44.9 (3.4)	53 (3.2)	37.5 (6.7)	41.2 (6.4)
At last visit	88		60.1 (2.8)	66.5 (1.3)	53 (7.5)	58.2 (5.2)
	Median (IQR)		Median (Q1-Q3)	Median (Q1-Q3)	Median (Q1-Q3)	Median (Q1-Q3)
^ WFR at baseline	0		0.05 (0- 0.12)	0.2 (0.03- 0.4)	0 (0- 0.1)	0 (0- 0.1)
WFR at follow-up 1	0		0 (0- 0.04)	0.03 (0- 0.06)	0 (0- 0.2)	0.05 (0- 0.06)
WFR at follow-up 2	0		0 (0- 0.04)	0 (0- 0.04)	0 (0- 0.1)	0 (0- 0.01)
^						
PFR at baseline	0		0 (0- 0.3)	0.11 (0- 0.3)	0 (0- 0.11)	0.13 (0- 0.4)
PFR at follow-up 1	0		0 (0- 0.1)	0 (0- 0.2)	0 (0- 0.12)	0 (0)
PFR at follow-up 2	0		0 (0- 0.14)	0 (0- 0.3)	0 (0- 0.4)	0 (0- 0.1)
USS at baseline	0		7 (5- 8)	4.5 (3- 5.25)	7 (6.25- 8.5)	8 (7- 8.5)
USS at follow-up 1	0		6 (5- 8)	5 (3.25- 6.25)	7 (5.25- 9.5)	7 (5- 8.5)
USS at follow-up 2	0		6 (5- 7)	5 (3.75- 6)	6.5 (6- 8.5)	7 (5.5- 8.5)
CODS at baseline	4		5 (2- 8)	5 (3.5- 6)	6 (2.5- 8)	7 (1- 8.5)
CODS at follow-up 1	10		7 (5- 8)	7 (4.75- 8)	8 (5.75- 8.75)	5(4- 8.5)
CODS at follow-up 2	5		6 (5- 8)	7 (3.75- 9.25)	7 (5.5- 7.75)	6 (4.5- 7.5)

SNOX; sialadenitis, nodal osteoarthritis & xerostomia, SS; Sjogren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, +; with another autoimmune disease, WFR; whole flow rate, PFR; parotid flow rate, USS ultrasound score, CODS; clinical oral dryness score. ^ (ml/min). Q1; first quartile, Q3; third quartile. Data are reported as median± IQR. (Kruskal-Wallis test followed by Dunn's post hoc test).

3.5 Discussion

In addressing the aims of this study, we hoped to gain answers to a number of questions. Can a marker differentiate between the different subgroups of SS and show a difference to other disease control groups? Can ultrasound scores predict development of MALT-L? Furthermore, is there a clinical parameter (marker) that is able to monitor the progression of Sjögren's syndrome over time and does SS show loss of glandular function over time?

3.5.1 Cross- sectional analysis

Xerostomia is one of the main clinical manifestations seen in SS patients. However, this subjective complaint may not be necessarily reflecting an actual hyposalivation and loss of salivary gland function (Billings et al., 2016). The loss of glandular function can be measured via overall or specific glandular flow rates, (CODS) or implied from glandular structure (USS). Histology would be the most suited test demonstrating the actual loss of normal tissue that results in functional loss but other than the difficulty of obtaining repeated samples (ethically) we have mentioned previously that functional loss might not be a result of tissue loss and patients with normal glands might manifest signs of functional loss. Thus, it was decided to select applicable tests that can be repeated easily while correlating selected measures with histology.

SNOX patients were enrolled in this study and the subsequent studies (chapters 4 and 5) as a non-SS sicca (disease) control group because the disease is often confused with SS and shares some symptoms; fatigue, dryness complaints and signs; inflammation of the salivary glands (non-specific vs. focal in SS groups). SNOX was little tested by other studies since its first description by Kassimos et al., (1997) and the following Price and Venables, (2002), it was interesting to follow this disease group further.

Regarding the MALT-L risk patients, a number of risk factors have been documented from large cohort studies (Papageorgiou et al., 2015a, Nishishinya et al., 2015). However, a clear

explanation as to why these factors should facilitate lymphoma development has not been clarified yet, besides they might be as a result of an advanced disease rather than a possible risk factors. For this study, in order to have more stringent selection with specified patients who are considered at risk, a group of at least 3 factors was chosen as a selection criterion, and patients were further sub grouped to high or low risk depending on the severity of the manifestations (factors). In chapter 1 subsection 1.6.4 the commonly reported factors were linked to high risk patients and those less reported (including the USS which is not commonly reported by others) were linked to low risk patients. Patients in this study demonstrated at least 4risk factors.

The MALT-L patients group included in this study was confirmed via biopsies of either parotid gland in most of the patients or minor salivary glands in 2 patients, while one patient had submandibular lymphoma. They were included for further verification where comparisons between them and MALT-risk groups as well as SS patients can be done efficiently.

3.5.1.1 Clinical parameters

The cross-sectional analysis part of this study has revealed a significant reduction of WFR in the overall SS group when compared to the disease control group. This is mostly attributed to the subgroups of MALT-L risk and MALT-L as they were the ones showing the greatest reduction when compared to the disease controls as well as to the SS subgroup (**figure 3-2**). Whole flow rate could not differentiate between SS subgroups and the other forms of dryness and this is supported by a number of studies (Billings et al., 2016, van den Berg et al., 2007, Osailan et al., 2012, Ohyama et al., 2015) although the last study demonstrated higher values but showed a similar trend. Normal healthy subjects were not included for this study due to a difficulty in finding healthy subjects data but a small group (n=20) were collected and included for the subsequent studies (chapters 4 and 5) and their flow rates were evaluated and compared to the rest of the present groups (disease controls and SS subgroups). Most studies have reported that reduced whole flow rates were frequently found in SS patients compared to healthy controls (Mandel and Baurmash, 1976, Skopouli et al., 1989, Sreebny and Zhu, 1996, Carpenter et al., 2000, Kalk et al., 2002a, Vissink et al., 2003, Helenius et al., 2005, Billings et al., 2016). In fact, it is one of AECG criteria (Vitali et al., 2002).

Similar findings were found regarding the PFR where the mean flow rate was reduced in comparison to the disease controls yet again it was mainly because of the MALT-L risk and MALT-L subgroups since no differences were found between the SS subgroup and the disease control and only the MALT-L group was reduced compared to the SS subgroup (**figure 3-3**). Again other similar findings were reported previously with similar values in disease control and SS subgroup (Kalk et al., 2001a, Kalk et al., 2002c, van den Berg et al., 2007, Osailan et al., 2012). Once more the differences between SS and other groups involved comparisons with healthy controls more frequently. This supported our findings from the next chapter (chapter 4) where we have included healthy controls as well. In a way, it also supported the findings from this chapter since a lot of our patients were considered to be at later stages of SS (with the inclusion of all of the at risk and MALT-L groups) showing reduced

parotid flow rates. Also, this was supported by others where they have reported reduced SS flows (Stuchell et al., 1984 , Kalk et al., 2001a, Kalk et al., 2002c, Pedersen et al., 2005).

Concerning CODS, the mean value of the overall SS group was greater than the disease control group and each of the subgroups was higher than the disease control while MALT-L was increased compared to the SS subgroup (**figure 3-5**). Similarly, Osailanet al., (2012) reported an increased CODS of SS patients when compared to SNOX and NOS while DIH was insignificantly lower than the SS scores (Challacombe et al., 2015).

The USS mean values of the overall SS group and the SS subgroups were significantly increased when compared to the disease control group. Furthermore, both MALT-L risk and MALT-L were higher than SS subgroup. USS was the only parameter that showed a difference between high and low MALT-L risk patients (**figures 3-4 and 3-6**).

3.5.1.2 Associations

Cross- sectional association (**table 3-2**) has identified a strong negative correlation between both salivary flow rates (whole and parotid) and CORDS most notable with the whole flow rate. A study has identified a similar finding supporting the present results. CORDS was the highest in the group of patients demonstrating the lowest flows (irrespective of their diagnosis) (Osailan et al., 2011, Osailan et al., 2012, Challacombe et al., 2015).

Both WFR and PFR showed a strong correlation ($r=0.6$, $p<0.0001$) in concordance to Kalk et al., (2002a) and Vissink et al., (2003) ($r=0.75$, $p<0.001$). Although studies reporting the involvement of parotid glands in later stages of SS suggest that there is no correlation between whole mouth and parotid flow rates and that the reduction of whole saliva is attributed to the significant decrease in the submandibular/sublingual flows (Vissink et al., 1993, Kalk et al., 2001a, Kalk et al., 2002c, van den Berg et al., 2007, Pijpe et al., 2007) but patients involved in this study are considered at their later stages specially those who are at risk or developed lymphoma and this was evident when the correlation was done for the subgroups individually where it was ($r=0.5$, $p<0.0001$) for SS while it was ($r=0.9$, $p<0.0001$) for MALT-L. Regarding the USS, again a significant negative correlation was found with salivary flow rates and a positive correlation with the CORDS so a more severe case (reflected by low flows and high CORDS) will tend to have a high USS, although the association is considered moderate and it will be discussed further in the following section.

It is well known that measurement of salivary flow (Jorkjend et al., 2004, Burlage et al., 2005) and presumably CORDS may show variations in the same individual and between other individuals although this has not been verified in this study but it should be noted for further investigation. Such variation needs to be studied in USS as well; inter- and intra- individual and observer variability should be considered.

3.5.1.3 Diagnostic accuracy of USS

The sensitivity of the USS ≥ 4 yielded 81.03% with a specificity of 94.3% and odds ratio of 70.5% in this study (**figure 3-7**). Two systematic reviews reported comparable results, one reported a lower sensitivity yet comparable specificity values. Delli et al., (2015) stated a pooled sensitivity of 69% (95%CI: 0.67–0.71), specificity of 92% (95%CI: 0.91–0.93), and diagnostic odds ratio of 33.89 (95%CI: 20.75–55.35) in their recent meta- analysis. Also, in their systematic review, Jousse-Joulin et al., (2016) have reported up to date studies evaluating the usefulness of ultrasound in which a number of them showed good sensitivity and specificity in 4 out of 11 studies with a range of (45.8 to 91.6%) sensitivity and (73 to 98.1%) specificity. Some studies has suggested adding USS to the objective measures of the AECG criteria as it showed an increased sensitivity up to 87% while retaining its specificity (Cornec et al., 2013). Others such as Bootsma et al, (2013) have recommended correlating it with flow rates of parotid and submandibular glands. Although the latter was not done in our study we can comment on the ultrasound score relation to the parotid flow rates. In our results both whole and parotid flow rate were correlated with USS (spearman $r = -0.4$, p value <0.0001) confirming its importance as a diagnostic test (**table 3-2**). The same paper also pointed out the potential of USS in identifying MALT-L, and this was done in our study where its ability to differentiate between different subgroups of SS as well as between other non- SS sicca patients was tested via roc curve analysis establishing the optimal cut off score that provided the maximum sum of sensitivity and specificity followed by calculating the negative and positive predictive values via contingency tables (**table 3-4**). The diagnostic odds ratio was calculated to measure the effectiveness of ultrasound as a diagnostic test enhancing its reliability which is also confirmed in our study via the significant correlation between the histology focus scores and ultrasound scores of the overall subjects (spearman $r = 0.7$, p value <0.0001) and a considerable association in the SS groups (spearman $r = 0.3$, p value <0.0001) thus suggesting a uniform disease process and supporting their other suggestion of using ultrasound as a first measure (Bootsma et al., 2013). Therefore, theoretically a positive result of an ultrasound (score of 4 or more) complemented by other tests such as ocular, serology

and flows would replace the need for performing a biopsy unless otherwise indicated, for example; in case of MALT-L suspicion.

The use of USS as a diagnostic test was confirmed by Astorri et al., (2016) reporting an agreement of 91% ($\kappa=0.82$) showing that USS had a positive predictive value of 85% and a negative predictive value of 96% of the histology results. In contrast to our results the level of agreement was lower showing 87.3% concordance between USS and focus score with a slightly higher (88.1%) positive predictive value but lower negative predictive value (89.6%) of the biopsy results.

However, one must bear in mind that although focus score is considered a gold standard test, it does not mean that it has 100% sensitivity and specificity as it might misclassify some patients introducing false positive and false negatives. Thus, the predictive values in the present study were calculated based on a given confirmatory diagnosis and there was a lower negative predictive value (66%) while the positive predictive value was higher (97%) than the values calculated in relation to histology results. So, although the number of false negatives was high (thus the lower NPV) we should note that some patients possibly in earlier stages of SS with score of 3 or less were considered as negatives but on the other hand it limited the number of false positives making it therefore a simple test to exclude patients without SS.

We report lower USS performance when differentiating between SS subgroups (**table 3-3**); sensitivity (range 70- 84%), specificity (range 74-98%), PPV (range 40-95%) and NPV (range 68-95%) being lower especially in the later subgroups with higher cut- offs that provided the maximum sum of sensitivity and specificity in each comparison. The lowest positive predictive values of 40% in case of cut- off ≥ 6 to differentiate between SS and SS at risk or with MALT-L are due to the presence of a high number of false positives which could for example be a patient with low risk showing no hypo-echoic foci shown by USS or in case of patient with MALT-L post- surgery who is scored accordingly with a lower USS but on the other hand the high NPV makes it difficult to include false negatives. USS could not differentiate between the MALT-L risk and MALT-L. Despite having high specificity values in discriminating SS from

other forms of dryness (NOS, SNOX and DIH), which generally presumed that they do not have that much of an effect on the structure of the glandular parenchyma. In this study 70 DCT patients were given an USS value and only 9 gave a score greater than zero while the rest were zero scored; only 4 of the 9 patients had scores of 4 or 5 making them false positives while the rest of the 9 patients had an average score of 3 as opposed to the advanced glandular involvement in SS. It should be considered that other conditions might cause a similar ultrasound picture and this needs further attention. Furthermore, it should be noted that the above-mentioned studies have reported different USS scoring systems, thus Jousse-Joulin et al., (2016) have highlighted the importance of developing a consensual scoring system with consistent procedures and appropriate training in achieving reproducible results. Moreover, similar to other researchers they have suggested that USS can be an early diagnostic test in SS diagnosis as it usually involves all of the glands opposing to sialogram that only gives information regarding a single gland. Although we have successfully attempted to correlate histology with ultrasound, one must not forget that the former deals with minor glands while the latter is related to major glands and it would be more appropriate to correlate it with major gland histology or vice versa (ultrasound of minor glands correlation with the focus scores) and that warrants further investigation.

Additionally, although it is not our main focus in this study but it is worth stating that the mean focus scores of the SS subgroups differed significantly from the control group being mostly 0 in the latter while the SS subgroups were showing a gradual increase in their focus score being 3.7, 5 and 6 in SS, at risk and MALT-L respectively.

The correlation between USS and flow rates indicates the association between the severity of sialadenitis seen as increased USS and functional incapacity of salivary glands, although it should be noted that it was a moderate correlation even if it was statistically significant.

From the cross-sectional study the following conclusions can be drawn; regarding whole and parotid flow rates, the overall SS group showed a significant difference when compared to the non- SS sicca disease controls. It could be due to the significant difference observed in both MALT-L risk and MALT-L groups since SS subgroup did not differ from the disease control group. As for the CORDS score and the ultrasound scores differences can be seen between the overall SS as well as the SS subgroup when compared to DCT. The two advanced groups (at risk and MALT-L) showed a significant difference when compared to SS subgroup as well, indicating the necessity of identifying and monitoring them.

USS would be the ideal non-invasive test to differentiate and monitor SS patients in general as proven in this study (81% sensitivity and 94% specificity) and supported by previous studies replacing sialography/ scintigraphy and showing a good correlation as well as agreement with focus scores, thus providing more evidence on its reliability. An optimum cut- off ultrasound score of 4 could differentiate SS from non- SS patients while higher scores are used for the later subgroups with a reduction in both sensitivity and specificity. However, this can be used to prioritise biopsy for patients showing evidence of SS in their ultrasound.

3.5.2 Follow- up studies

The longitudinal follow-up part of this study (either 5 or 10 year) has shown that SS patients did not change significantly regarding WFR, PFR, USS and CODS indicating a stable and rather a mild course of SS disease where the duration has not influenced the salivary gland function (**tables 3-5 and 3-7**). There are many possible factors that might affect the exocrine function as mentioned before in chapter 1 subsection 1.4.1 but in this present study we have not focused on them since our primary outcome was investigating the effect of time on salivary function.

3.5.2.1 Five-year follow- up

Few studies have investigated the long-term course of SS and similar findings were reported by some of them as well; 39 SS patients were followed prospectively by Jonsson et al., (1993) over a period of 3.25 years (39 months) where histology focus scores and WFR (stimulated) were re-evaluated. They have reported no statistically significant changes in the salivary flow values while their focus scores were increased.

Theander et al., (2005) reported non- significant deterioration of WFR in their cohort of SS patients (n=58) over 5 years (prospective follow- up), stating that 40% of their patients were either 0 or 0.1 mL/ 15 min at baseline thus having this low volume will not become any further reduced. This supported our results; 40% had 0 WFR and 32% had reduced WFR (<0.1mL/min) and this was due to the inclusion of SS at risk and MALT-L groups. Furthermore, in the present study, patients with 0 WFR remained the same while those with reduced WFR became 0 and only some of them (n=6) were improved showing WFR >0.1 mL/min. The rest of the patients who had normal flow rates at baseline remained stable except for reduction in 3 patients. According to Theander et al., (2005) 7% of the patients had improved while 33% had worsened. Despite those similarities, our means were lower than their reported means (0.8 mL/min at baseline and became 0.7 mL/ min) reported by them while ours was 0.1 mL/min and became 0.07 mL/min, this is due to the fact that in our cohort, patients at risk or with MALT-L were included as well. They have used a visual analogue scale (VAS) for oral dryness

and reported deterioration in some of the patients and improvement in others (Theander et al., 2005). Whereas CODS in our cohort showed a non- significant reduction where 22% of patients with low CODS became worse while the rest did not (few were improved and some were stable). Most of the patients with high CODS remained stable as well.

Pijpe et al., (2007) followed 60 SS patients prospectively recording patients' whole and glandular saliva flow rates at baseline and follow- up along with sialochemistry analysis. Similarly, unstimulated WFR (as well as unstimulated glandular flow rates) remained at their rather low readings while glandular saliva (stimulated PFR) was correlated with disease duration; decreased flows at follow- up, thus indicating that there is a loss of stimulated glandular function over time which contrasted our findings. Pijpe et al., (2007) found that this reduction is even more prominent in submandibular/sublingual flow rates especially in the early phases of SS. Furthermore, they reported no difference between SS patients with or without another autoimmune disease, nor between the VAS of oral dryness at baseline and follow- up which are similar findings to ours. Another follow- up study was done on SS patients (n=43) and reported similar findings (change in flows at later visit) (Bouma et al., 2003). A possible explanation for the controversy regarding the PFR is the inclusion of SS patients with an early onset disease in their study vs. late stages in ours.

Yet again, (Haldorsen et al., 2008) reported similar findings supporting our results; a median 5- year retrospective follow- up of 141 patients has revealed a non- significant change in the median WFR stating a reduction in 29.8% of patients and increase in 31.9%.

In our study, out of the 73 patients 29 had an initial PFR of 0 which didn't change in most of them (5 only were improved), 6 patients had flows of ≤ 0.2 mL/min, 4 became worse, one did not change and one was improved. While in patients with ≥ 0.2 mL/min, 15 showed worsening of their flows and only 3 were improved while the rest did not change.

3.5.2.2 Ten- year follow- up

Regarding the 10-year follow- up, similar findings were found; these preliminary results indicate no change in salivary function over time and they are in accordance with the results of a Japanese study which investigated signs and symptoms of 43 SS patients reporting no progression of sicca symptoms over 10-20 years period (Cui et al., 1997).

Relative stability of SS patient's signs and symptoms as well as for their serological markers was stated over a 5 to 10-year period - up, reporting the development of extra- glandular manifestations in some of their patients (Gannot et al., 2000). The stimulated PFR of most of the patients remained the same with some elevation. Although the results might be misleading as some of the patients did not show salivary function on their initial visit and thus it cannot become any worse. However, other patients' flow rates did not change overtime as seen in the previous studies as well as in ours indicating that a compromised salivary function can be relatively stable over years.

Haga et al., (2002) have studied the association between low WFR and different clinical and immunological markers including SS duration (mean 13.5 years) reporting no significant correlation between them. This was explained by the inclusion of a relatively high number of patients with long disease duration. However it was noted that the WFR of almost half of his patients was > 1.5 ml/15 min (Pijpe et al., 2003).

3.5.2.3 Other considerations in follow-up studies

Finding records of a 5- year follow- up of SNOX patients was challenging while 10-year follow-up was impossible (one patient) since they were normally discharged following confirmation of their diagnosis or followed for few years. When they were re-invited, we had to consider their age which hindered them from coming, thus it was difficult to confirm the stability of this disease over the 10-year period. However, no change was noted over 5 years among all the measured parameters confirming the stability and low progressing nature of it. The one patient included in the 10-year study showed no salivary flows despite showing normal glands on the ultrasound and her CODS was considered moderate at her first and last and severe in between visits.

Other points worth mentioning are:

1. Some patients were recorded since their date of experiencing dryness symptoms not per their diagnosis which was confirmed a few years later following their initial visit while some were referred following their diagnosis from other hospitals.
2. Regarding SS patients with MALT-L the data suggests that a larger group of patients were in the 5- year category. However, many of the others who were recently diagnosed (or seen), would have already attended different clinics (rheumatology clinics for example) and been diagnosed by others earlier and then referred to Oral Medicine clinics later.
3. The medical records of number of patients were up to 20 years old but USS were only introduced in 2007 which limited our search up to only 10 years and only 16 patients with full records were included. In addition, digital ultrasound images were not found in order to score them retrospectively (only few were done when found).
4. Despite the high variability of the groups it can be concluded that there is no significant decline in exocrine gland function over time regarding WFR, PFR, USS and CODS while individual patients may display flare ups and remissions as in any other

rheumatic diseases. The small number of patients involved in the 10-year follow-up study precludes definitive conclusions but it was in accordance with other studies.

5. The subjective complaint of xerostomia is not always associated with an actual decreased flow as demonstrated in this chapter especially in the disease control group and SS subgroup where the median whole flow rate was 0.1 mL/min and almost 0.1 mL/min respectively while median parotid flow rate was 0.3 mL/min and 0.2 mL/min in the cross-sectional part of this study. Xerostomia might be associated with compositional changes of saliva (Carpenter et al., 2000, Kalk et al., 2001a, Kalk et al., 2002c, Helenius et al., 2005, Pedersen et al., 2005, Billings et al., 2016). Thus, the next 2 chapters we will be focusing on the compositional changes of saliva stating their relevant studies as well.

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4 Salivary proteomic biomarkers in Sjögren's syndrome

4.1 Introduction

The identification of the protein profile of body fluids, such as saliva, is considered one of the promising strategies for the discovery of new biomarkers. There is a well-recognized need for the identification and validation of biomarkers to possibly be used in diagnosis, prognostic assessments, as a research tool and probably to identify novel targets for therapeutic intervention. Saliva can be non-invasively collected, thus providing an attractive alternative approach that harbours a wide range of components. Changes in these components are assumed to reflect the pathogenesis of Sjögren's syndrome (SS), because salivary glands are the major site of autoimmune destruction, saliva may represent the most appropriate body fluid for biomarkers identification.

In addition, there is a 1000-fold increased risk of lymphoma development in parotid glands from SS patients compared to other NHL subtypes (Ekstrom Smedby et al., 2008). This makes parotid saliva a valuable substrate that might be able to differentiate between the different subgroups of SS patients (SS, SS at risk of developing lymphoma and those with lymphoma). Several proteomic studies have shown differential protein expression in the saliva of SS patients and healthy control subjects (Fleissig et al., 2009, Ryu et al., 2006, Hu et al., 2007b, Ambatipudi et al., 2012, Deutsch et al., 2015, Giusti et al., 2007, Baldini et al., 2011b). Some studies have validated their candidate markers (Hu et al., 2010). While few have performed proteomic analysis of samples from lymphoma patients (Baldini et al., 2011a, Hu et al., 2009). In this study, proteomic analysis of parotid saliva samples from healthy subjects, SS patients and SS patients at risk of developing lymphoma was done. This was followed by selecting a candidate marker and validating it via ELISA on a larger group of samples including disease control (SNOX) patients and SS patients who have developed lymphoma. The marker was further tested on whole mouth saliva and differences between both types of saliva were compared.

4.2 Aims and objectives

It is of great importance to assess whether saliva proteomic biomarkers can be used for the stratification of patients with Sjögren's syndrome (SS).

In this study, it was hypothesized that SS related proteins exist in human saliva and could be used to discriminate SS patients from healthy control subjects. Furthermore, identified salivary biomarkers could be useful in identifying a subgroup of SS who may progress to lymphoma.

To address this, the following aims were under taken:

1. Analyse newly collected saliva samples from SS patients to determine salivary biomarkers of disease activity.
2. Analyse newly collected saliva samples from SS patients who are at risk of developing lymphoma to determine salivary biomarkers associated with development of lymphoma.

Selection of a candidate protein for initial biomarker verification is followed and evaluation of its predictive value for the detection of SS, SS at risk of developing MALT-L and MALT-L was commenced.

The specific aims were as follows:

3. Investigate the potential use of the candidate protein as a diagnostic biomarker in discriminating SS with different subgroups.
 - a) Compare the levels of this protein in SS patients to levels in healthy and diseased control patients (SNOX).
 - b) Establish whether there is an association between the candidate biomarker with the overall salivary gland function.

4.3 Materials and methods

4.3.1 Study group

Six, age- and sex- matched subjects (2 healthy controls, 2 SS and 2 SS at risk of developing MALT-L) were included in the proteomic analysis.

For further analysis via ELISA, their samples were then added to a larger group of subjects with a total number of 83. Diagnosis breakdown for subjects was healthy controls, diseased controls (SNOX), SS groups combined and separated (SS, SS at risk of developing MALT-L and those with MALT-L).

4.3.2 Saliva sample collection

Stimulated parotid saliva (n=83) and unstimulated whole mouth saliva samples (n=56) were collected and stored as outlined in chapter 2, subsection 2.1.4. A minimum of 1 hour fasting preceded all sample collections. Their flows and total protein concentration were measured as described in (**Appendix 4 & 5**) and chapter 2, subsection 2.2.1.

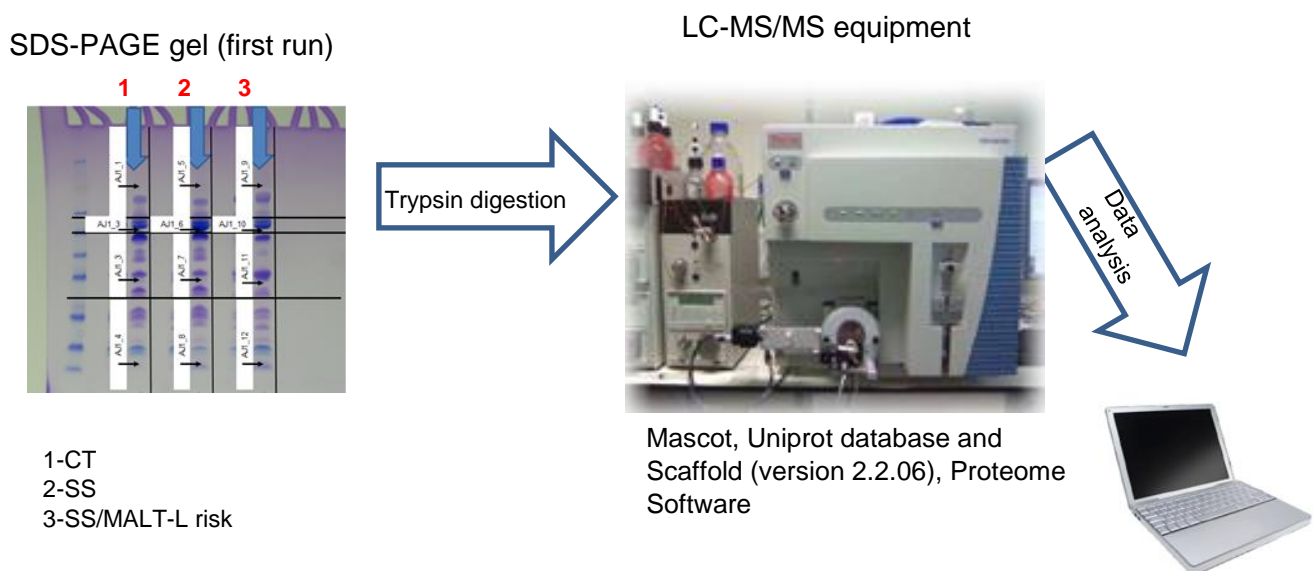


Figure 4-1 Schematic representation work flow of the protein identification for parotid saliva samples based on 1D gel electrophoresis, trypsin digestion, LC-MS/MS and database searching

4.3.3 Laboratory analyses

4.3.3.1 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

4.3.3.1.1 SDS-gel electrophoresis and total protein stain

Two sets of parotid saliva samples (gels) (n=3 each) were sent for proteomic analysis separately in different occasions (different runs), they were prepared under reducing conditions and subjected to gel electrophoresis then stained as described earlier in chapter 2, subsections 2.2.2 and 2.2.3.

4.3.3.1.2 LC-MS/MS and database searching

The two sets of gels (each with 3 bands representing the different groups) were excised to four sections and treated as described in chapter 2, subsection 2.2.4. The section with the high abundant protein amylase was taken and analysed at the end, to enhance the detection of the low abundant proteins (**figure 4-1**). All LC-MS/MS data were searched using the MASCOT database search algorithm as mentioned previously. The results were then analysed separately and introduced as the first proteomic analysis (run) and the second run.

4.3.3.2 ELISA

Commercially available pre-coated plates (Quantikine ELISA Kit, R&D Systems, Minneapolis, MN), (cat. no. DS8900) for S100A8/S100A9 Heterodimer were used for both parotid saliva (n=83) and whole saliva (n=57). Detailed protocols are found in chapter 2, subsection 2.2.5 and (**Appendix 6**).

4.3.4 Statistical analysis

All results were exported to Windows® Excel 2007 spread sheet. Proteomics data were compared using fold change ratios and fold change while the statistical analyses for ELISA data, were carried out via SPSS. GraphPad Prism® 6 (GraphPad Software Inc., California, USA) was used for the graphical presentation.

4.4 Results

4.4.1 Proteomic analysis

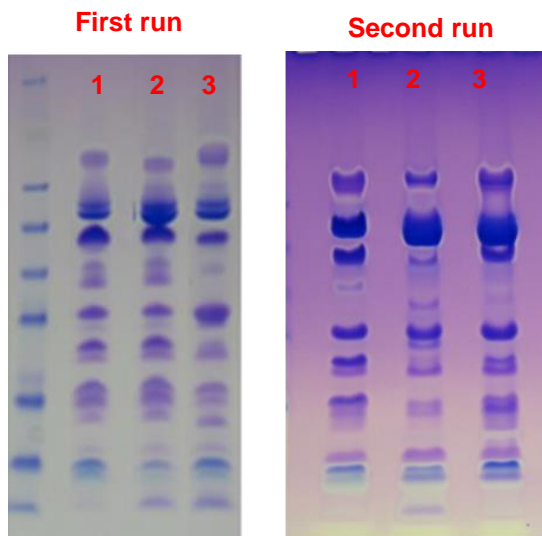


Figure 4-2 Representative two SDS-PAGE gels of parotid saliva samples from healthy controls and Sjögren's syndrome patients.

1; healthy controls, 2; SS, 3; SS at risk of developing mucosa associated lymphoid tissue lymphoma (MALT-L), all sent for proteomic analysis.

4.4.1.1 Study group

Table 4-1 Demographics of patients included in the salivary proteomic analysis

Parotid saliva analysis	First run	Second run
Age*	42.3 ± 2.5	51.7 ± 1.7
Gender	F	F
Diagnosis	1 CT 1 SS 1 SS at risk	1 CT 1 SS 1 SS at risk
Count	3	3

*Age difference is due to sample availability by time of collection.

4.4.1.2 LC-MS/MS database searching

A total of 1324 proteins were identified overall from the first run (at lowest stringency; when parameters (filters) were set to their minimum values), while 818 proteins were detected from the second run. These proteins were filtered with these parameters; minimum number of 3 peptides, meeting or exceeding protein threshold values for 95% confidence level and 80% peptide threshold, resulting in 161 proteins and 144 proteins respectively for each run. The identified proteins were sorted into functional categories based on their annotations in the Uniprot database and Gene Ontology searching (**figure 4-4**). Both were further filtered by choosing the immune system process category, which resulted in 53 proteins in the first run and 45 in the second as marked on (**figure 4-4**). The relative expression levels (fold change ratio) of these proteins were compared between control samples and SS at risk of developing MALT-L samples at first then between control samples against SS samples. A secondary comparison of proteins was done after logarithmic transformation of the raw ratios (\log_2). A final comparison between both runs was performed following their individual analysis where differences between both disease samples were identified. Work flow is summarised in (**figure 4-3**).

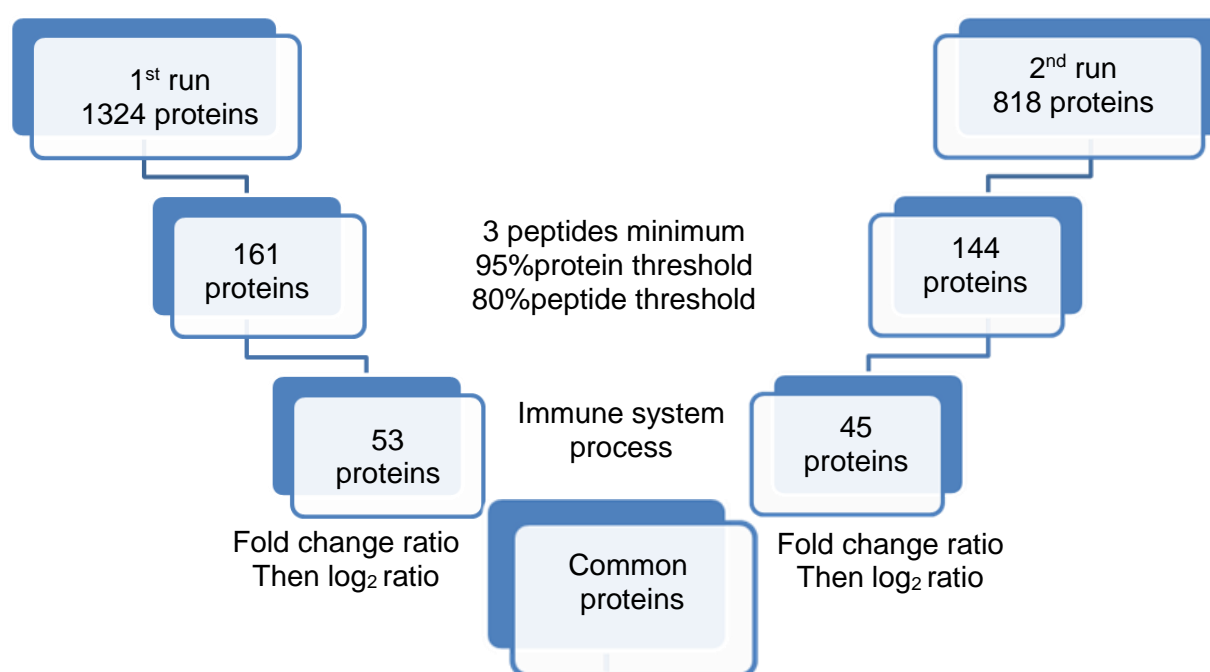


Figure 4-3 Schematic presentation of LC-MS/MS database searching of both runs

Starting with an arbitrary fold change ratio cut- off of ≥ 2 , the proteins were further reduced to 45 and 17 proteins in the 1st and 2nd run correspondingly. An example of the Scaffold list of identified proteins of the second run and their peptide count is shown in (**figure 4-5**). After comparing both runs, the preliminary list of common proteins of interest were selected (n=11) as shown on (**figure 4-5**). **Appendix 9 & 10** demonstrate these proteins with their total ion currents (TIC) and their fold change ratio between sample groups calculated by TIC for the first and second runs. In addition, 6 of them showed up-regulation in SS samples when compared to controls in both runs.

For the first run, some proteins were present only in the SS at risk of developing MALT lymphoma patient but neither in the healthy control nor the SS patients (e.g. Matrilysin). Proteins not identified in healthy control but present in both diseased patients were 14-3-3 protein zeta/delta, Thioredoxin and Protein S100-A9. The remaining proteins had an increased fold change in both diseased patients compared to the healthy control. Among them, Annexin A1, Actin cytoplasmic 2, Ig γ -1 chain C region and S100-A8 showed the highest fold changes. Similarly, the same eleven proteins were expressed differently between the samples in the second run. Some proteins were not present in the control sample but present in both samples from the disease groups (e.g. 14-3-3 protein zeta/delta and Matrilysin. The rest again had high fold change values in samples from disease groups.

The list of proteins was further narrowed; the ratios between SS at risk of MALT-L to healthy controls were transformed to \log_2 values to have more stringent selection with a cut- off set to 2. Likewise, the differences between the control samples and SS samples were detected. The final selection was done based on having ≥ 2 fold increase in both diseased samples (SS and SS at risk of MALT-L) as well. Thus, four proteins; Actin cytoplasmic 2, Ig γ -1 chain C region, S100-A8, and S100- A9 were finally selected (**figure 4-6**). One of which was not present in healthy sample of the first run. A final comparison was done between both disease samples of each run as shown in **table 4-2**.

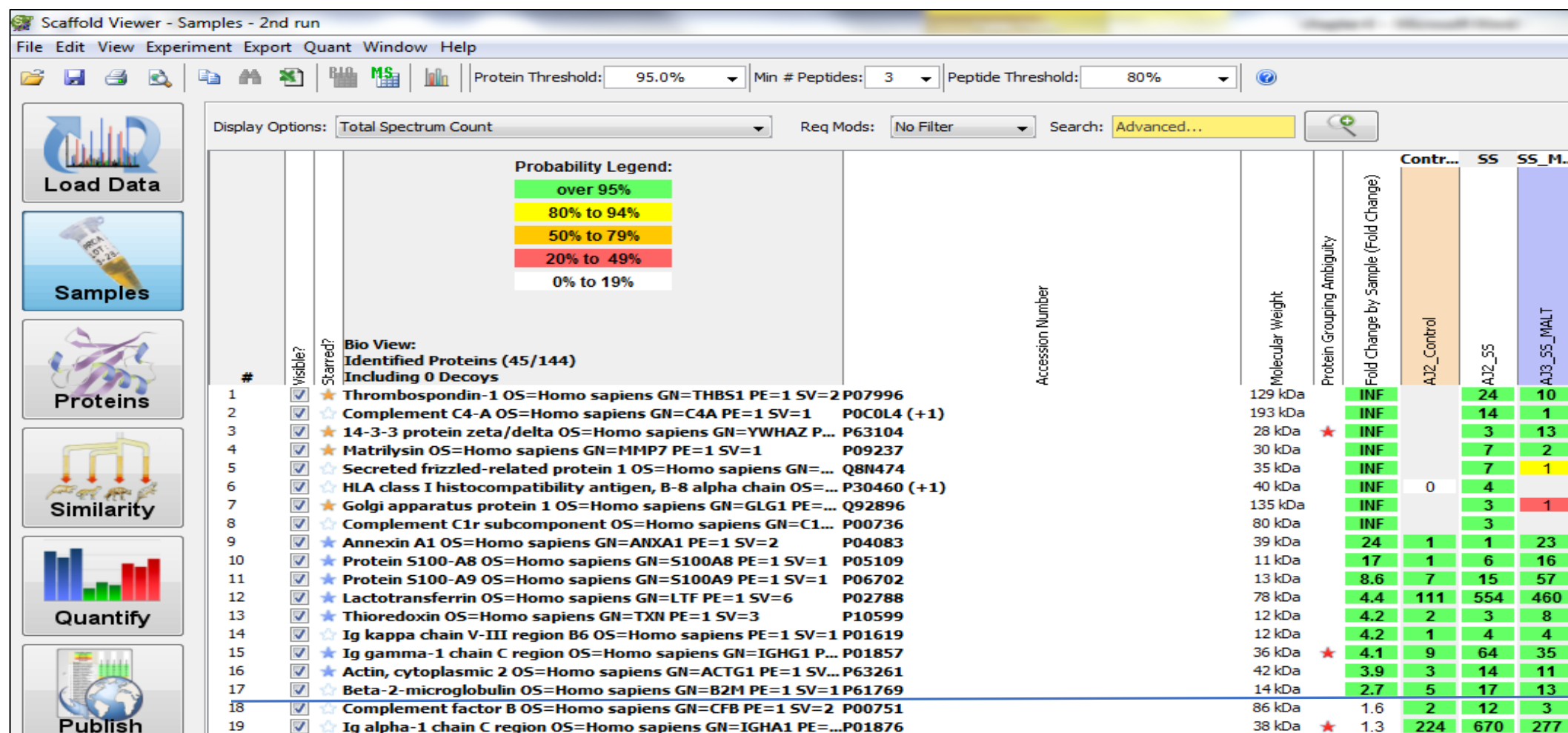


Figure 4-5 Scaffold list of identified proteins and their peptide count of the second run

SS at risk of lymphoma development was compared to healthy control using arbitrary fold change ratio cut-off of ≥ 2 , the proteins were selected as determined by the blue line ($n=17$). Further to comparison with the first run, the common proteins were identified as marked on this graph; Yellow starred are the proteins not present in the control sample but present in both disease samples. Blue starred are the proteins increased fold change in the diseased samples compared to the control sample

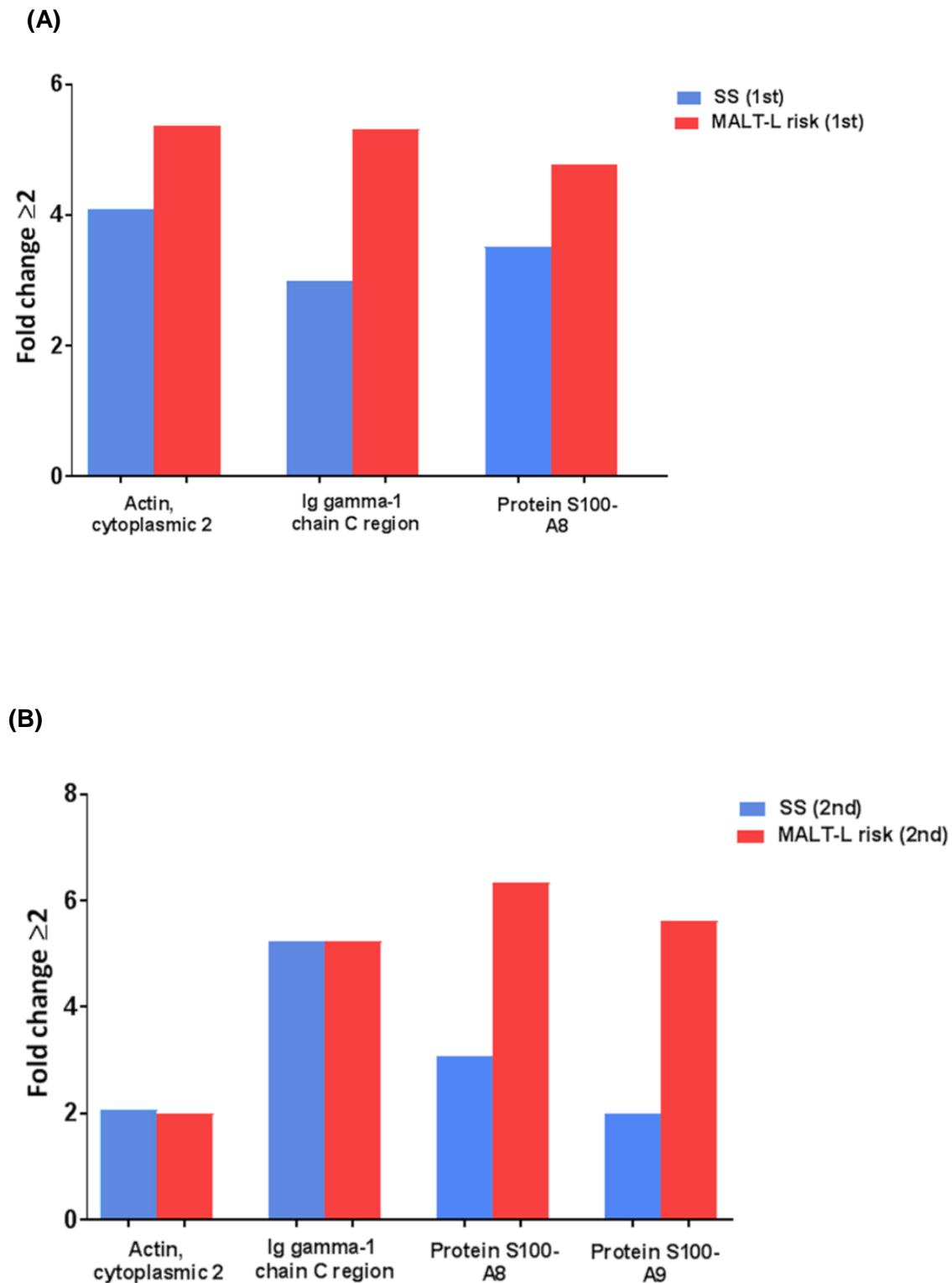


Figure 4-6 Final proteins selected using additional fold change (\log_2) cut-off of 2 or more when compared to controls.

(A) Three proteins in the first run, protein S100-A9 was not detected in the control sample.

(B) Four proteins in the second run.

SS; Sjögren's syndrome, MALT-L risk; SS patients at risk of developing mucosa associated lymphoid tissue lymphoma.

Table 4-2 Final proteins with their fold change ratio and fold change when SS patients at risk of developing MALT-L are compared to SS patients

MALT-L risk/ SS	First run		Second run	
	Fold change ratio	Fold change Log₂	Fold change ratio	Fold change Log₂
Actin, cytoplasmic 2	2.4	1.3	1	0
Ig γ-1 chain C region	5	2.3	1.004	0.01
Protein S100-A8	2.4	1.3	9.6	3.3
Protein S100-A9	2.1	1.1	12.1	3.6

SS; Sjögren's syndrome, MALT-L risk; SS patients at risk of developing mucosa associated lymphoid tissue lymphoma.

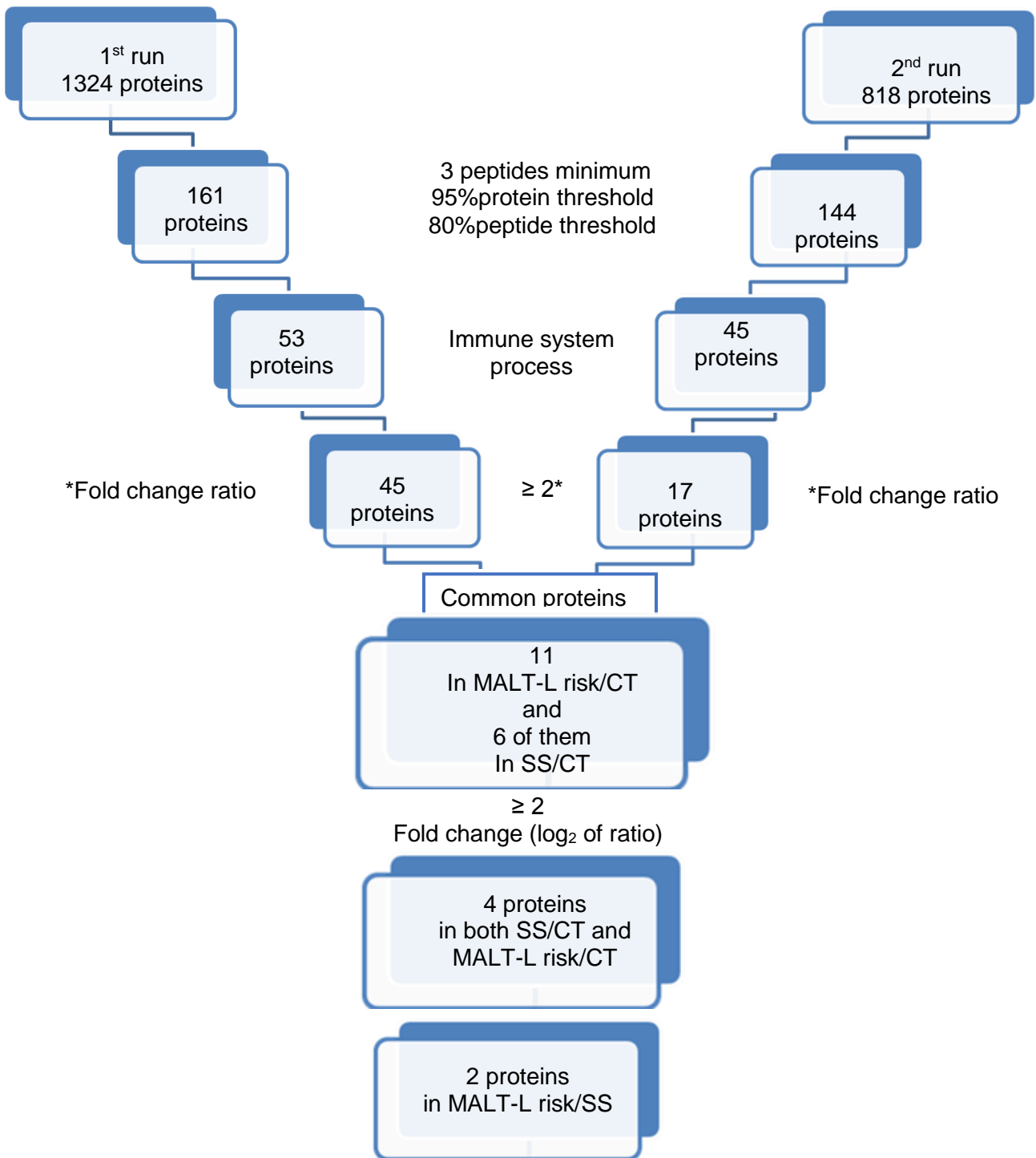


Figure 4-7 Overall schematic presentation of LC-MS/MS database searching and analysis of both runs

4.4.2 Preclinical validation by immunoassay (ELISA)

4.4.2.1 Study group

A total of 83 subjects' parotid saliva samples were included for this study, summary of patients' demographics and clinical data is shown in **table 4-3**. As part of their routine clinical assessment, parotid and whole mouth saliva flow rates were collected and recorded.

4.4.2.2 Salivary flow rates and the total protein concentration

Whole mouth saliva (WMS) was collected from 56/83 (68%), insufficient samples were mostly from the patients group; SS 11/19 (57%), SS at risk of developing cancer 8/18 (44%), SS who developed the cancer (SS/MALT-L) 7/14 (50%) and SNOX 12/14 (85%). Total protein concentrations were assayed for all, except for one WMS sample and one parotid saliva sample.

Whole flow rate (WFR) was significantly lower in SS patients (overall cohort) compared to controls ($p < 0.0001$). While it was lower in SS patients at risk of lymphoma development and those who had already developed it ($p = 0.001$ and $p = 0.01$, respectively) in comparison to controls (**figure 4-8**). Stimulated parotid flow rate (PFR) was significantly lower in SS/ MALT-L patients compared to healthy controls ($p = 0.024$) even though the overall SS group did not differ significantly (**figure 4-9**). Total protein concentration for whole saliva was higher in the overall SS patients compared to the disease control group ($p = 0.016$) and it was higher for only the patients who developed the lymphoma in comparison to the disease controls ($p = 0.026$). On the other hand, the total parotid protein concentration did not differ significantly between the groups (**figures 4-10 and 4-11**).

A negative correlation was found between whole mouth saliva flow rate and total protein concentration (Spearman $r = -0.34$, $p = 0.012$) while the parotid flow rate and total protein concentration were not correlated. No association was found between whole mouth saliva and parotid flow rates nor between their total protein concentrations (**table 4-4**).

Table 4-3 Demographics and clinical characteristics of the SS patients and control subjects included in ELISA

Controls					Patients									
Diagnosis	Healthy controls (CT)		Disease controls (SNOX)		SS		SS at risk		SS/ MALT-L		Overall SS		Statistically significant	
Total (n= 86)	Median (Q1-Q3)	n	Median (Q1-Q3)	n	Median (Q1-Q3)	n	Median (Q1-Q3)	n	Median (Q1-Q3)	n	Median (Q1-Q3)	n	Groups	p value
Age (years)	50.7±2.6	19	62.7±2.6	15	51.5 ±2.7	20	51.9± 3	18	53.7± 2.6	14	52.3±1.6	52		
sex	F	18		15		19		18		14		51		
	M	1		0		1		0		0		1		

Whole saliva

WFR (n=56)	(ml/min)	0.31 (0.12- 0.54)	18	0.11 (0.08- 0.3)	12	0.11 (0.01- 0.24)	11	0.005* (0- 0.12)	8	0.04* (0- 0.17)	7	0.08* (0- 0.2)	26	CT---Overall SS	<0.0001
														CT---at risk	0.001
														CT---SS/MALT-L	0.01
Total protein (n=55)	(mg/ml)	1.3 (0.94-1.6)	18	1.04 (0.64- 1.6)	12	1.3 (0.91- 2.9)	11	3.2 (1.66- 3.5)	8	3.2* (2.1- 7.2)	6	2.4* (1.14- 3.5)	25	SNOX---Overall SS	0.016
														SNOX---SS/MALT-L	0.026

Parotid saliva

PFR (n=83)	(ml/min)	0.33 (0.2- 0.45)	18	0.29 (0.13- 0.44)	14	0.23 (0.2- 0.52)	19	0.2 (0.11- 0.47)	18	0.08* (0- 0.25)	14	0.21 (0.085- 0.44)	51	CT---SS/MALT-L	0.024
Total protein (n=82)	(mg/ml)	0.87 (0.46- 1.7)	18	0.88 (0.48-1.4)	14	1.3 (0.68- 2.5)	19	1.02 (0.7- 1.3)	18	1.3 (0.92- 1.5)	13	1.2 (0.73- 1.5)	50		NS

CT; healthy controls, SNOX; sialadenitis, nodular osteoarthritis and xerostomia, SS; Sjogren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, at risk of developing MALT-L, WFR; whole flow rate, PFR; parotid flow rate. Data are reported as Median and Q1; first quartile, Q3; third quartile. (Kruskal-Wallis test followed by Dunn's post hoc test).

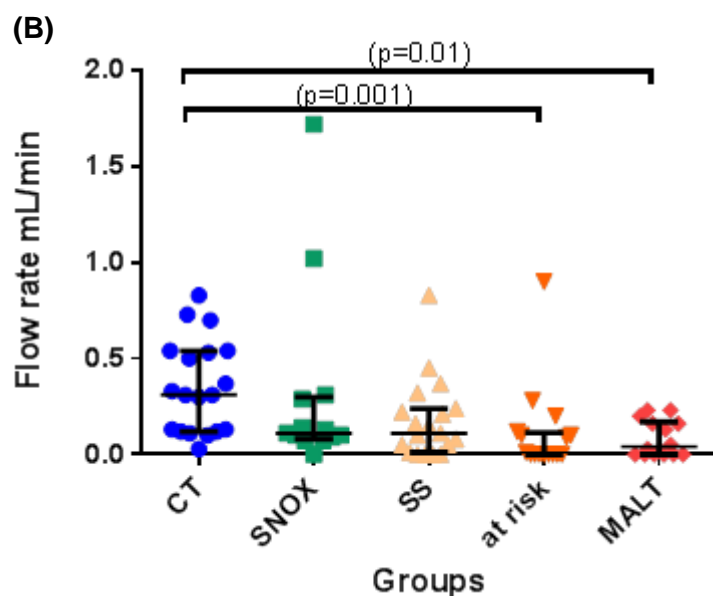
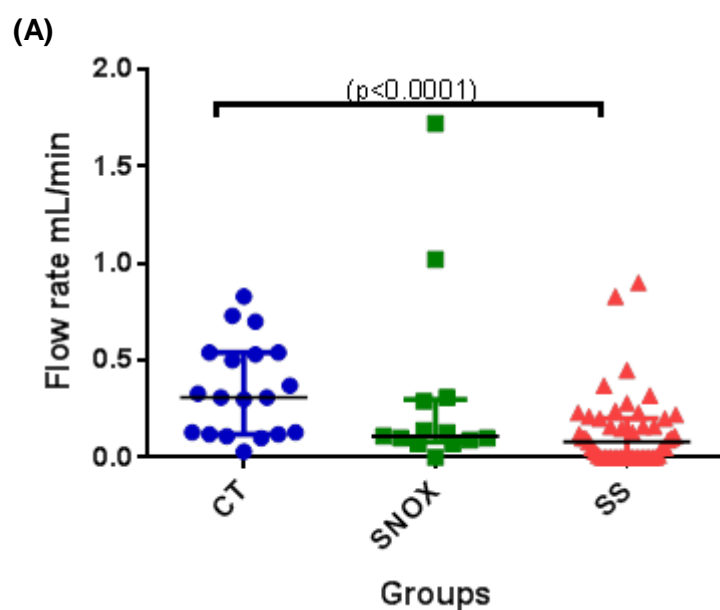


Figure 4-8 Whole mouth saliva flow rates of different groups (A) SS groups are combined (B) SS subgroups. (n=56)

CT; healthy controls, SNOX; sialadenitis, nodular osteoarthritis and xerostomia, SS; Sjogren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, at risk; at risk of MALT-L. Data are reported as median \pm (IQR) and expressed as mL/min. (Kruskal-Wallis test followed by Dunn's post hoc test).

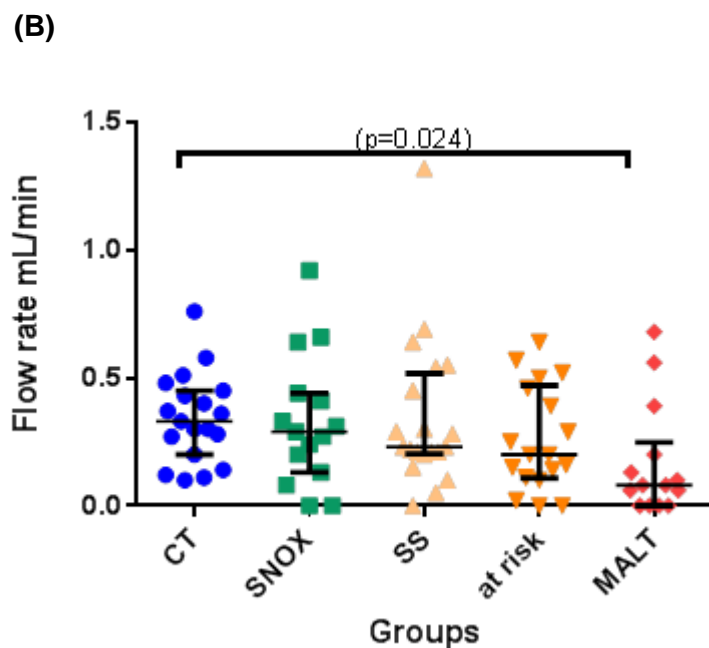
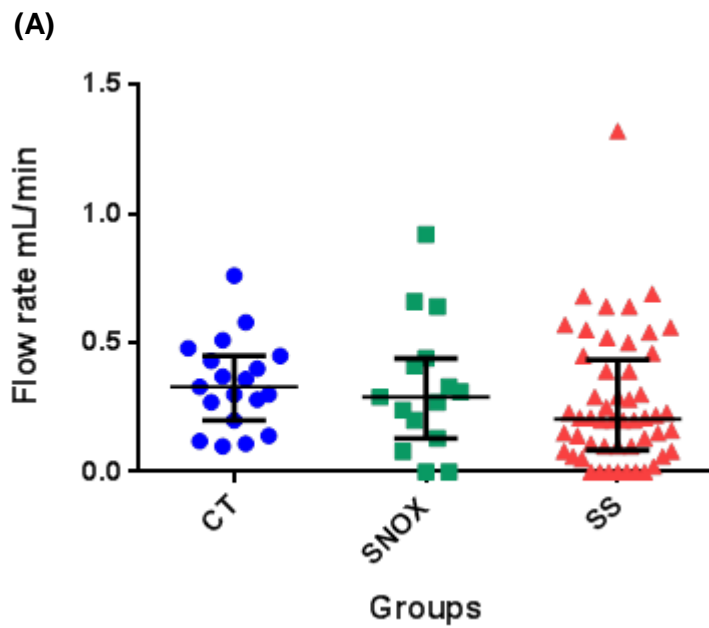


Figure 4-9 Parotid saliva flow rates of different groups (A)SS groups are combined (B) SS subgroups. (n=83)

CT; healthy controls, SNOX; sialadenitis, nodular osteoarthritis and xerostomia, SS; Sjogren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, at risk; at risk of MALT-L. Data are reported as median \pm (IQR) and expressed as mL/min.

(Kruskal-Wallis test followed by Dunn's post hoc test).

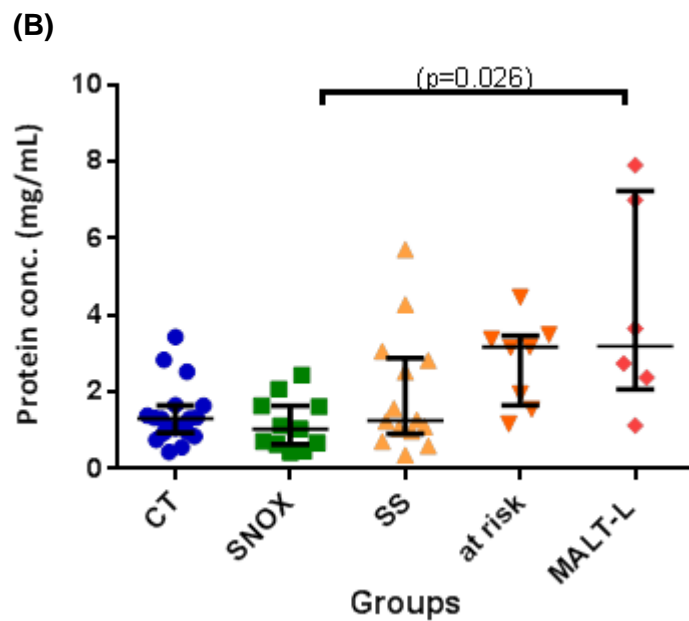
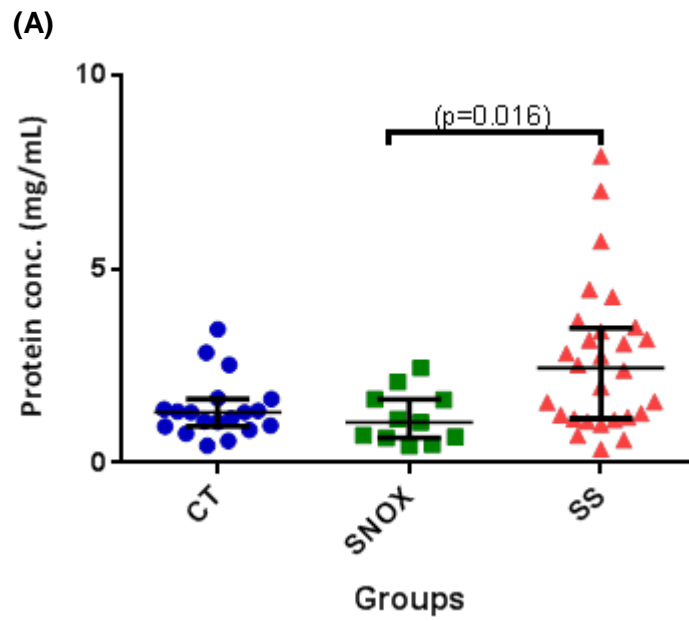


Figure 4-10 Total whole protein concentration of different groups (A) SS groups are combined (B) SS subgroups. (n=55)

CT; healthy controls, SNOX; sialadenitis, nodular osteoarthritis and xerostomia, SS; Sjogren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, at risk; at risk of MALT-L. Data are reported as median \pm (IQR) and expressed as mg/mL. (Kruskal-Wallis test followed by Dunn's post hoc test).

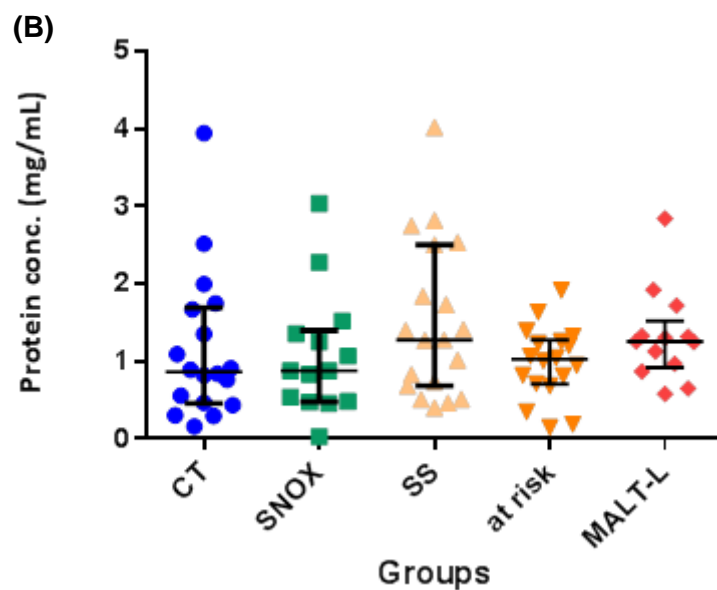
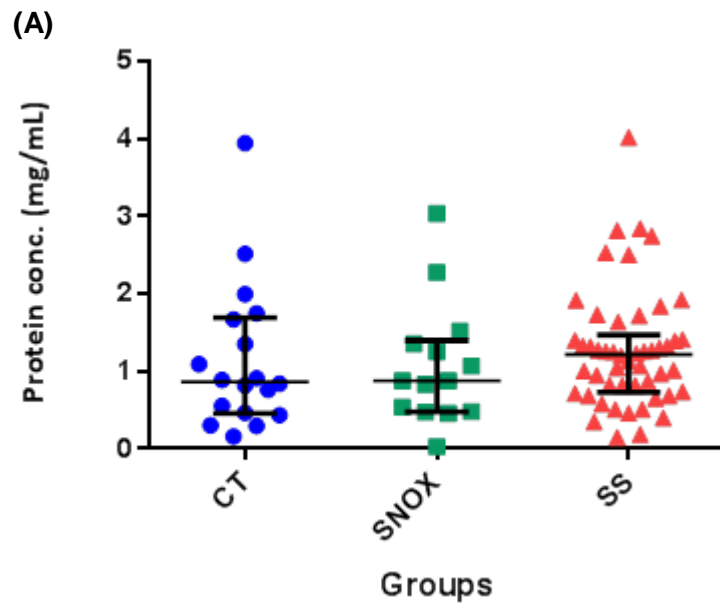


Figure 4-11 Total parotid protein concentration of different groups (A) SS groups are combined (B) SS subgroups. (n=82)

CT; healthy controls, SNOX; sialadenitis, nodular osteoarthritis and xerostomia, SS; Sjogren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, at risk; at risk of MALT-L. Data are reported as median \pm (IQR) and expressed as mg/mL. (Kruskal-Wallis test followed by Dunn's post hoc test).

4.4.2.3 Association of the flow rates and total protein concentration

Table 4-4 Spearman correlation between flow rate and total protein concentration of parotid and whole mouth saliva

Spearman's rho				
	WFR	PFR	TWPC	TPPC
WFR	1			
PFR	0.1	1		
TWPC	-.34*	-0.06	1	
TPPC	-0.02	-0.03	0.07	1

WFR; whole flow rate, PFR; parotid flow rate, TWPC; total whole protein concentration, TPPC; total parotid protein concentration. *P <0.05

4.4.2.4 Salivary S100A8/A9 levels

Table 4-5 Additional clinical characteristics of SS patients

	SS subgroups n/n* (%)	SS n/n* (%)	SS at risk of MALT-L n/n* (%)	SS with MALT-L n/n* (%)
Other autoimmune disease	19/51 (37)	7/19 (37) 2 RA 5 SLE	8/18 (44) 2 RA 5 SLE 1PBC	4/14 (29) 1 RA 3 SLE
Treated with immunosuppressive agents	38/51 (75)	-	-	-
At high risk	-	-	11/18 (61)	-

n*; overall number of group, SS; Sjogren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, RA; Rheumatoid arthritis, SLE; systemic lupus erythematosus, PBC; primary biliary cirrhosis.

4.4.2.4.1 Parotid saliva

The median concentration (Q1-Q3) of S100A8/A9 in parotid saliva from overall SS patients was 743.1 (91- 3526) ng/mL while the healthy and disease controls were 31.9 (0- 273.2) and 208.9(0- 265.3) ng/mL respectively (**figure 4-12 (A)**). Significant differences were found in S100A8/A9 levels of the overall SS patients group compared to healthy and diseased controls groups ($p=0.001$ and 0.031) correspondingly.

When comparing the subgroups of SS patients; median concentration of S100A8/A9 in SS patients was 506 (84.6- 1031) whereas it was 666.8 (131.2- 5642) and 937.4 (90- 4566) for SS patients at risk and those who already developed the cancer respectively. Both later groups showed a significant difference when compared to the healthy control group; ($p=0.014$) for the patients who already developed the cancer and ($p=0.019$) for those who are at risk of developing it while insignificant increase was noted in SS subgroup (**figure 4-12 (B)**). Visible differences were noted during the ELISA (**figure 4-13**).

4.4.2.4.2 Whole mouth saliva

Although higher median level was detected in the overall SS group 16628 (3165- 20184), it was not statistically different from the healthy and disease groups 8457(687.3- 17154) and 3857 (2418- 8697) (**figure 4-14 (A)**).

However, SS subgroups did show significant higher levels; median concentration of patients who already developed lymphoma 22545 (16032- 28096) was higher than the healthy control group ($p=0.046$), SNOX group ($p=0.009$) and even the SS group 5244 (1550- 17225) ($p=0.049$). SS patients who are at risk of developing lymphoma had high levels 18841 (4034- 19872) but not statistically significant (**figure 4-14 (B)**).

All of the variables were statistically analysed using Kruskal-Wallis One-Way Analysis Of Variance (ANOVA) followed by post-hoc Dunn's test for multiple comparisons. An example of the SPSS analysis on parotid saliva is available in **Appendix 11 &12**.

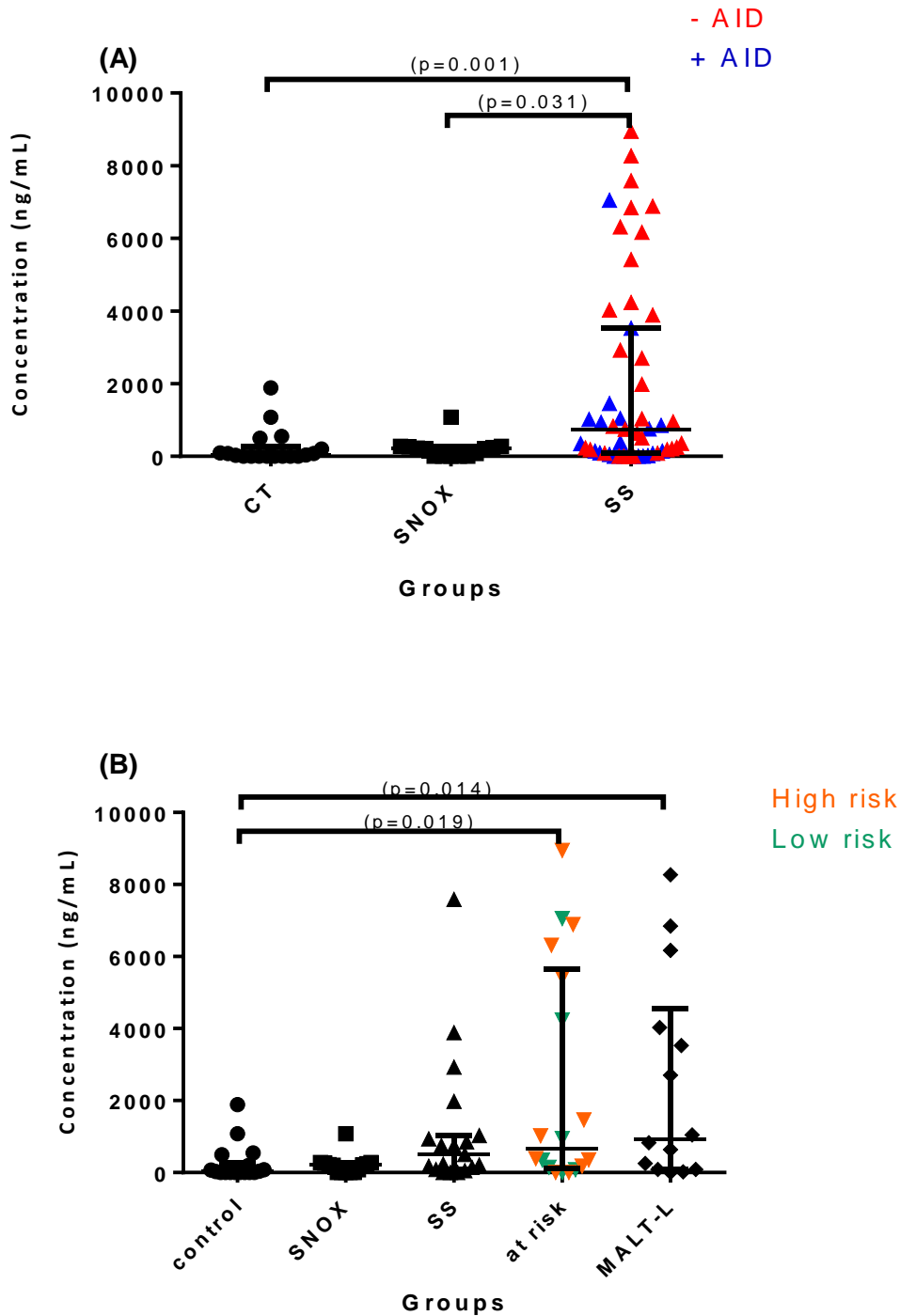


Figure 4-12 Concentration of S100A8/A9 in parotid saliva from different groups (A) SS groups are combined (B) SS subgroups. (n=83).

CT; healthy controls, SNOX; sialadenitis, nodular osteoarthritis and xerostomia, SS; Sjogren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, at risk; at risk of MALT-L, +/- with or without, AID; autoimmune disease. Data are reported as median \pm (IQR) and expressed as ng/mL. (Kruskal-Wallis test followed by Dunn's post hoc test).

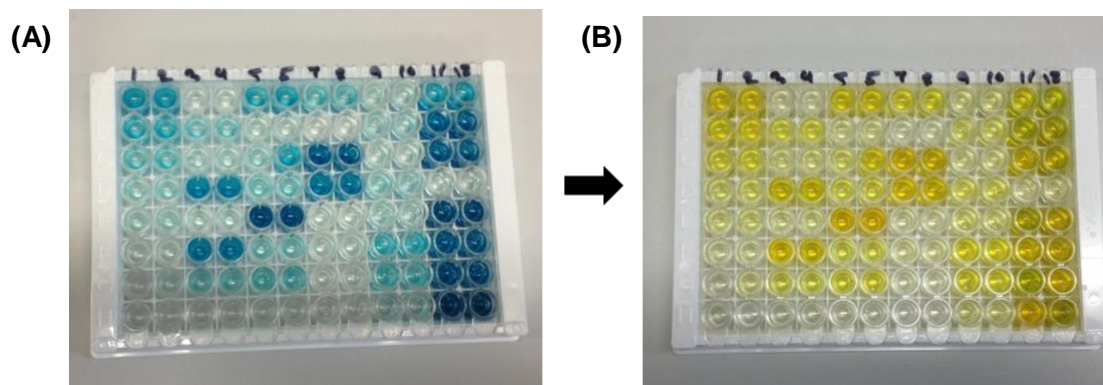


Figure 4-13 TMB substrate colour development of parotid saliva assay

(A) Before stop solution

(B) After stop solution

Obvious colour intensification is noticed towards the SS groups (lanes 7-12) specially the MALT-L group (lanes 11, 12).

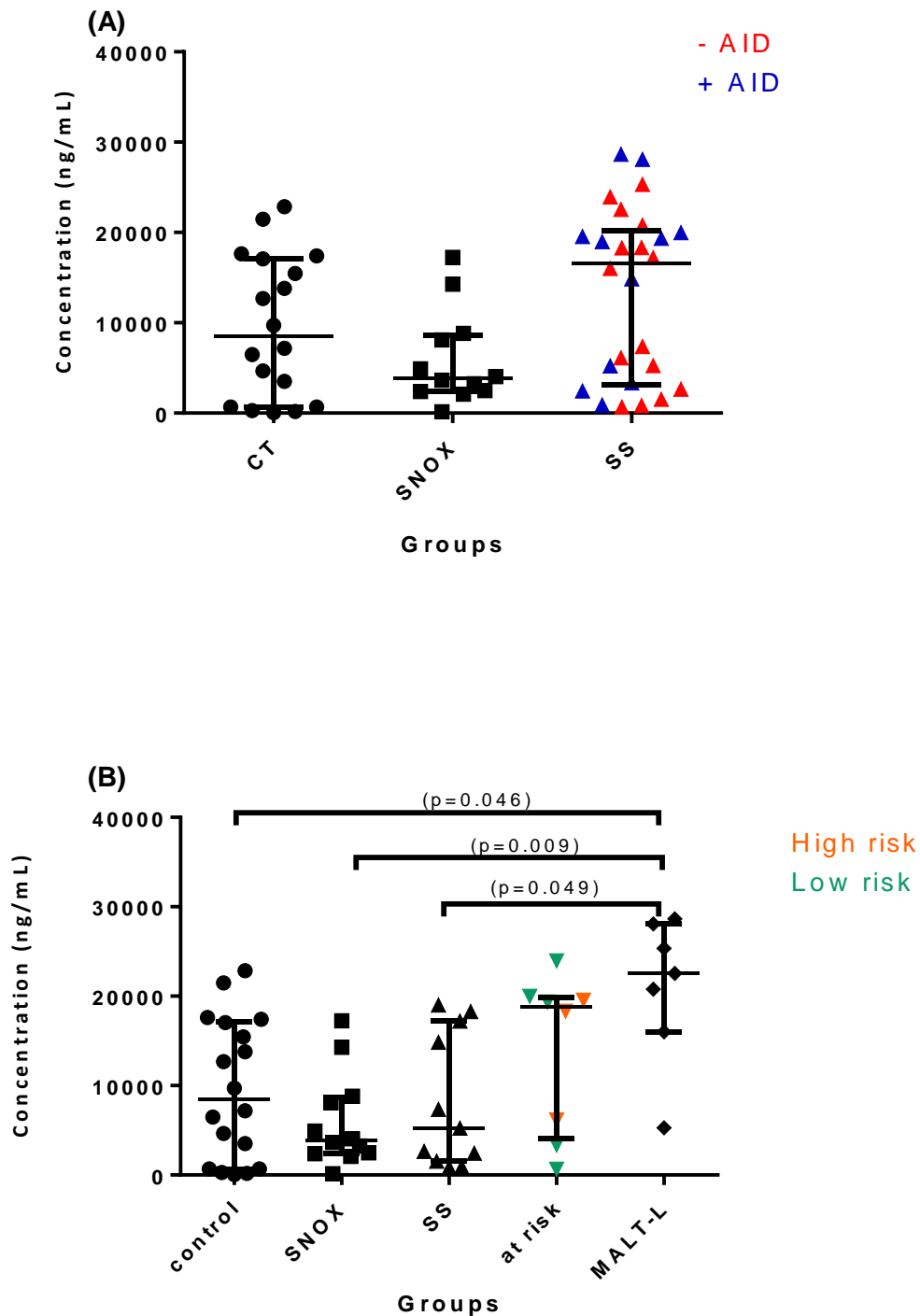


Figure 4-14 Concentration of S100A8/A9 in whole saliva from different groups (A) SS groups are combined (B) SS subgroups. (n=56)
 CT; healthy controls, SNOX; sialadenitis, nodular osteoarthritis and xerostomia, SS; Sjogren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, at risk; at risk of MALT-L, -/+ with or without, AID; autoimmune disease.
 Data are reported as median \pm (IQR) and expressed as ng/mL.
 (Kruskal-Wallis test followed by Dunn's post hoc test).

In both parotid and whole saliva, there was no statistically significant difference detected between patients with (red) or without (blue) another autoimmune disease (AID) (**figures 4-12 and 4-14 (A)**) nor between treated/ untreated patients as well (refer to **Appendix 13**). No differences were detected between patients at high (orange) and low risk (green) of developing lymphoma (**figures 4-12 and 4-14 (B)**) (Mann–Whitney U test).

Twenty- one samples (potential outliers) were repeated due to their questionable values; as zero (9 samples; 8 parotid samples and 1 whole sample) or high values (12 samples; 5 whole and 7 parotid samples), out of the 9 samples 5 remained zero while 4 gave a reading although not high, while all of the high readings gave similar values upon repetition.

The mean concentration of S100A8/A9 was compared between the right and left parotid glands of the same patient (n=2). Patients at risk of developing the cancer were selected where one gland shown with swelling while the other did not. Higher levels were detected in the swollen side for both patients (**table 4-6**). The higher levels were re-assayed as mentioned earlier and they gave similar results, therefore their means were taken and compared.

Table 4-6 Levels of S100A8/A9 in saliva from right and left side parotids

Concentration (ng/mL)		
	Mean of repeated patient1	not repeated patient2
Swollen parotid gland	9093.3	1103
Normal parotid gland	3550.3	467.6

4.4.2.4.3 Sensitivity of S100A8/ A9 immunoassay

The accuracy of the ELISA was tested for whole and parotid saliva by spiking samples with a known concentration of S100A8/A9. A four-point standard curve was generated with 4 dilutions (neat, 1:2, 1:4, 1:8) and compared to them (refer to chapter 2, subsection 2.2.5). The spiking test was done to check whether any salivary components such as mucins were inhibiting or interfering with the analyte detection. The results showed that the technique remained sensitive with the presence of saliva as there was no difference between the samples and the standard curve (all three lines representing the various conditions overlap) (figure 4-15). The mean recovery (accuracy) of the three samples was 107.7% ranged between 80.5 and 142.9%. The linearity was reported by the manufacturer (spiked whole mouth saliva) (n=4) with a range of 92-115% for dilutions 1:2, 1:4, 1:8 and 1:16.

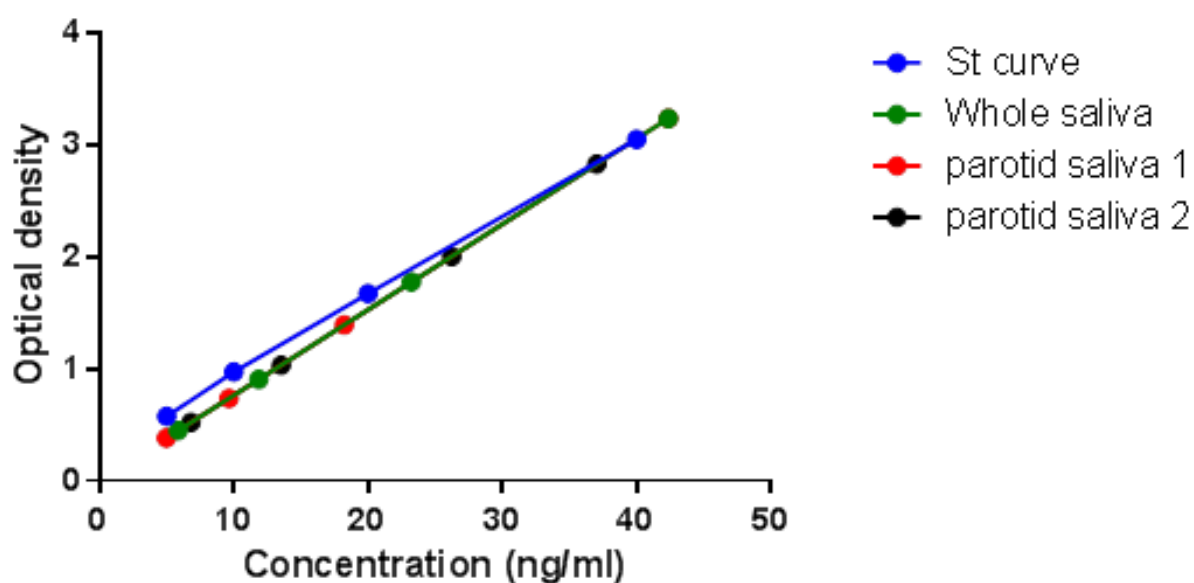


Figure 4-15 Observed concentration S100A8/A9 in spiked saliva samples (n=3)

Whole and 2 parotid saliva samples spiked 1:2 with highest calibrator and serially diluted.

4.4.2.4.4 Assay reproducibility

The reproducibility (intra-assay precision) of the ELISA was determined as the mean coefficient of variation (CV) of 5 replicates of 3 samples (positive parotid saliva) in one assay **table 4-7**. While the inter-assay precision was 8.2 % using two samples tested on 6 separate plates, these were comparable to the values reported by the manufacturer (2.7- 4.5% for the intra assay CV and 3.2- 5.8% for the inter assay CV).

Table 4-7 Intra-assay precision

	Sample1	Sample2	Sample3	Overall mean
Measurements (n)	5	5	5	-
Mean (ng/ml)	3550.3	7268.9	2034.4	4284.5
Standard deviation	124.6	313.9	59.6	166.03
CV (%)	3.5	4.3	2.9	3.9

4.4.2.5 Associations of S100A8/A9 levels

4.4.2.5.1 Whole and parotid saliva levels

There was a positive correlation between the salivary levels of S100A8/A9 in parotid and whole saliva of the overall subjects group (n=56) (spearman $r=0.268$, $p=0.046$) (**figure 4-16**). Correlation was found when SS subgroups were only included (n=26) (Spearman $r=0.496$, $p=0.01$). No association was found regarding the control group (n=18) (Spearman $r=0.106$, $p=0.67$).

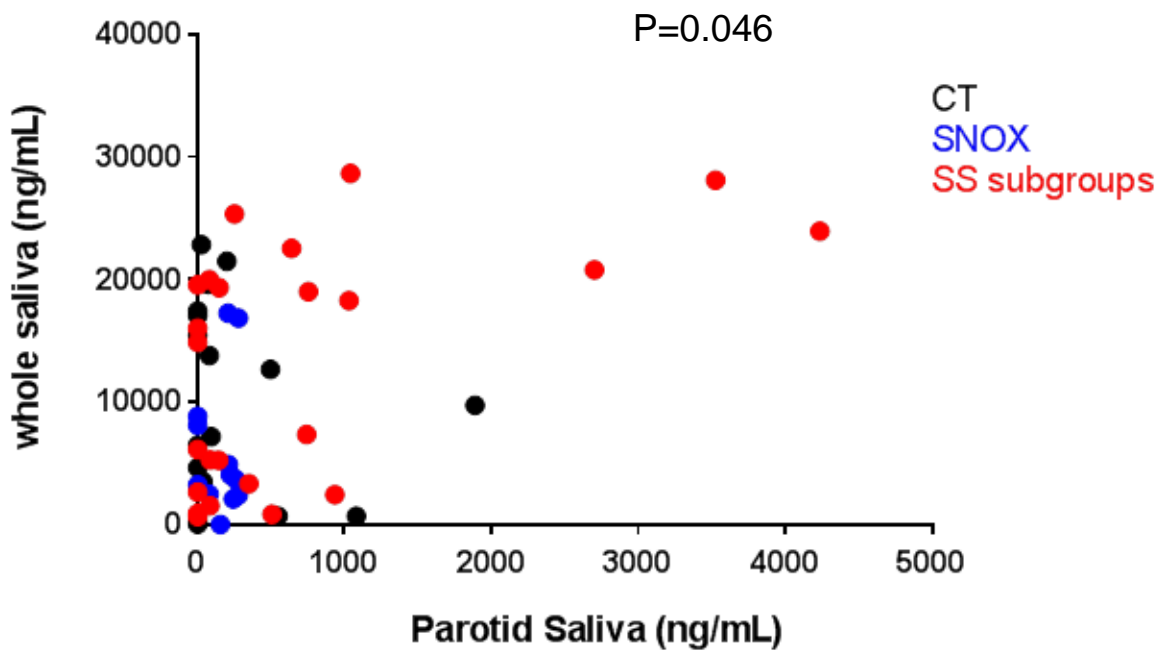


Figure 4-16 Spearman rank correlations of S100A8/A9 levels from parotid and whole saliva of all subjects (n=56)

4.4.2.5.2 In relation to clinical parameters

Correlation was examined between S100A8/A9 levels and 3 clinical parameters which are whole and parotid saliva flow rates and ultrasound scores. No correlation was noted between the parameters except for the parotid flow rate and calprotectin levels in parotid saliva as shown in **table 4-8**.

Table 4-8 Correlation between S100A8/A9 levels and different clinical parameters

Spearman's rho			
	N	Correlation Coefficient (r)	P value
Parotid			
PFR vs. S100A8/A9 levels	83	-0.256*	0.02
USS vs. S100A8/A9 levels	51	0.23	0.09
Whole			
WFR vs. S100A8/A9 levels	56	-0.138	0.309
USS vs. S100A8/A9 levels	26	0.14	0.495

PFR; parotid flow rate, WFR; whole flow rate, USS; ultrasound score. *P <0.05.

4.4.2.6 Receiver operator characteristic (ROC) curve of S100A8/A9 levels

ROC curves were constructed and showed that S100A8/A9 in parotid saliva was able to differentiate between control subjects and the overall SS patients with 82.35 % sensitivity (95%CI; 69.13- 91.6) and 56.25% specificity (95%CI; 37.66- 73.64) at a cut-off salivary concentration of ≥ 80.6 ng/ml (AUC=0.75 (95%CI; 0.65-0.85), $p=0.0001$). While in whole saliva the cut- off was ≥ 50766 ng/ml with 73.08% sensitivity (95%CI; 52.21- 88.43) and 50% specificity (95%CI; 31.3- 68.7) (AUC=0.73, (95%CI; 0.53- 0.82), $p=0.02$).

When comparing SS subgroups vs. controls only whole saliva levels could differentiate between SS with MALT-L and controls with a cut- off of ≥ 15748 and 85.71 % sensitivity (95%CI; 42.13% to 99.64%) and 80% specificity (95%CI; 61.43- 92.29) (AUC=0.89, (95%CI; 0.75- 1.026), $p=0.002$).

Whereas whole mouth saliva levels cut- off of ≥ 15449 ng/ml was able to differentiate between SS patients and SS patients who developed MALT-L with a 85.71 % sensitivity (95%CI; 42.13- 99.64) and 72.73% specificity (95%CI; 39.03- 93.98) (AUC=0.78, (95%CI; 0.74- 1.05), $p=0.006$).

4.5 Discussion

4.5.1 Proteomic analysis

This study examined whether differentially expressed parotid salivary proteins might serve as biomarkers of disease activity and be used for the stratification of patients with SS. The proteomics data generated by LC-MS/MS analysis suggested that a range of proteins including Actin cytoplasmic 2, Ig γ -1 chain C region, S100-A8, and S100-A9 have considerable potential as biomarkers and two of these proteins were selected for further validation via ELISA (**figure 4-6**) .

Ideally three patients per group should have been selected and analysed by LC-MS/MS at the same time for a proper comparison utilizing sound statistical methods (p- values). This was not achieved due to number of factors. Most importantly was the limited number of samples at the beginning of the study since MALT-L and MALT-L risk patients were initially difficult to identify with certainty. Secondly, since proteomics requires significant resources it was decided to use proteomic analysis as a guide to plan for a further validation experiment utilizing high throughput immunoassay to be done on a larger group of patients.

Two sets of samples each with 3 samples; 1 healthy, 1 SS and 1 SS at risk of MALT-L were analysed on different occasions due to their availability by time of analysis and their results were therefore not combined due to potential technical variations between runs.

Fold change ratio was calculated based on the total ion current in order to have unbiased values reflecting what has been actually measured using the mass spectrometer rather than using the peptide count.

Comparing SS at risk of developing MALT-L to healthy control sample has identified 45 over expressed proteins in the first run and 17 proteins in the second run. 22 proteins were found up-regulated when SS sample was compared to healthy control in the first run while 15 were identified in the second run (**figures 4-5 and 4-7**).

Attention should be taken in interpreting the results that some proteins were only seen in one group (either SS or SS at risk of MALT-L) but not in healthy control group, this might be due under sampling of low abundance proteins by MS.

There were no proteins with a fold change ratio of ≤ 0.5 of the first run when sample of SS patient at risk of MALT-L was compared to SS sample or to control sample but only 5 proteins had a decreased fold change when SS sample was compared to healthy control. On the other hand, the second run has revealed 11 down-regulated proteins when SS was compared to healthy and 17 were identified when patient at risk of MALT-L was compared to healthy control sample while 19 proteins had a decreased fold change ratio when samples from at risk patient was compared to SS sample.

It is important to note that due to the small number of patient samples this comparison is relative and does have possible errors thus data can't be normalised. The list of proteins had to be subjected to a more stringent criterion using an additional comparison method through logging the protein ratios which further reduced the proteins from eleven to four (Actin cytoplasmic 2, Ig γ -1 chain C region, S100-A8, and S100-A9) (**figure 4-6**).

This final selection has an increased fold change of at least 2 in both SS and MALT-L risk patients when compared to control samples in both runs. Only S100-A9 was not detected in control sample but was present in both diseased samples of the first run. Actin and Ig γ -1 chain C region had an increased fold change in the first run while protein S100-A8 and A9 had a higher fold change in the second run when SS patients at risk of developing lymphoma was compared to SS patients (**table 4-2**).

Due to differences in proteomics analysis techniques and variations in saliva collection methods, it might be difficult to compare results from different studies. As a general rule, whole mouth saliva (WMS) and parotid saliva (PS), can lead to different proteomics profile results; thus, different biomarkers are expressed. This does not seem to be the case for at least SS reported in a review by an American group comparing 5 studies 3 of which used WMS while 2 used PS and reporting similar biomarker findings among them (Al-Tarawneh et al., 2011). However, it is reasonable to say that whole mouth saliva will be more informative combining

different sources of secretion. On the other hand, the levels of many analytes are higher in the general circulation than in saliva and if blood was to leak into saliva, it will affect the actual levels of salivary analytes.

Also, stimulating parotid saliva may be required to obtain sufficient amounts of saliva (uncontaminated with blood), in cases where salivary flow is diminished such as SS thus providing more specific information regarding the gland itself (Al-Tarawneh et al., 2011).

Table 4-9 summarises more relevant information on those 5 proteomic studies of saliva together with our results. All the studies have compared SS to healthy controls. Moreover, one of these studies has included non-SS sicca syndrome and SS patients with other autoimmune diseases (Baldini et al., 2011b). Another study has comprised dry mouth patients (Ryu et al., 2006).

A recent study by Deutsch et al., (2015) analysing pooled WMS saliva from SS and healthy control patients. Amongst them Ig γ -1chain C region and S100-A8 were reported with an increased fold change ratio which corresponds well to our findings.

In another study conducted using comparable methods to those applied in the present study, pooled parotid saliva samples from 5 SS subjects and 5 healthy controls were analysed using LC-MS/MS. Among the identified proteins, Actin cytoplasmic 2, S100-A8, and S100- A9 were present supporting our results (Ambatipudi et al., 2012).

With regards to proteomic analysis on SS/MALT-L patients, none have investigated subjects at risk of developing MALT-L, but in a case report by Baldini and colleagues, a correlation was reported between the salivary proteomic profile and the clinical course of the disease in a patient with SS and B-cell lymphoma (MALT-L) for the first time. Changes in the levels of several types of immunoglobulin levels including Ig alpha-1 chain C region were detected (Baldini et al., 2011a).

In another study conducted by Hu and colleagues, parotid samples from SS patients and SS with MALT-L were analysed and compared to non-SS control subjects. They have found that many of the up-regulated proteins in SS/MALT-L and SS patients were related to immune responses and actin was amongst them (Hu et al., 2009).

Since the first proteomic study on parotid saliva of SS patients in 1999 by Beeley and Khoo (1999), saliva proteomics remained one of the most promising approaches to human disease biomarker identification with definite advantages in terms of non-invasiveness. It is important to note that stability of a normal saliva proteome is unknown and the characterization of it is of great importance. Furthermore, standardization of sample handling and collection is still required together with combining different proteomic analyses to achieve more reliable results and a significant effort, especially for validating salivary biomarkers, is still needed. Also, it would be of interest to perform an integrated proteomic analysis of the glandular tissue and compare it to saliva proteome results.

Finally, it is worth noting that this study is small thus the results should be regarded as preliminary and would be considered as a proof of concept thus it was followed by a larger (ELISA) study. And it would be of great significance to conduct the same comparison on larger group of patients using the same proteomic analysis to check for the reproducibility of the results. However, it is important to mention that most of the preliminary proteins have been associated with SS in one way or another in the literature (**table 4-9**).

Table 4-9 Studies of salivary proteome in SS including our results

Study references						
	Baldini et al., 2011b	Ryu et al., 2006	Giusti et al., 2007	Hu et al., 2007	Fleissig et al., 2009	Study results
Samples	UWMS	SPS	UWMS	SWMS SPS SSMLS	UWMS	SPS
Groups	19SS 21CT 10 nonSS 25SecSS	41SS 15 DM 5 CT	12 SS 12CT	10SS 10CT	16SS 8CT	2SS 2CT 2SS/MALT-L risk
Methods	2DE, MALDI-TOF	2D-DIGE, SELDI-TOF	2DE, MALDI-TOF	2DE, MALDI-TOF, LC-MS/MS	ESI-MS/MS	SDS-PAGE, LC-MS/MS
Identified proteins						
Actin, cytoplasmic 2	✓ ₁		✓ ₁	✓ ₂	✓ ₁	✓
Ig γ-1 chain C region				✓	✓	✓
Protein S100-A8				✓ ₃	✓	✓
Protein S100-A9	✓	✓	✓	✓	✓	✓

UWMS; unstimulated whole mouth saliva, SPS; stimulated parotid saliva, SWSF; stimulated whole mouth saliva, SSMLS; stimulated submandibular and sublingual saliva, SS; Sjögren's syndrome, CT; healthy controls, non SS; sicca patients not SS, SecSS; SS patients with other autoimmune disease, DM; dry mouth patients, 2D DIGE; two-dimensional difference gel electrophoresis, SELDI-TOF; surface enhanced laser desorption/ionization time-of-flight mass spectrometry, 2DE, two-dimensional gel electrophoresis, MALDI-TOF; matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, HPLC; high-performance liquid chromatography, ESI; electrospray ionization, LC-MS; liquid chromatography-mass spectrometry, MS/MS; Tandem mass spectrometry.

1. Alpha and Beta Actins
2. Alpha Actin
3. Down regulated

Modified from Katsiogiannis and Wong, (2016) and Al-Tarawneh et al., (2011).

4.5.1.1 Candidate biomarker selection

Choosing the candidate protein was challenging as all of them have been previously reported in SS literature (**table 4-9**) and all of them are up-regulated in both disease groups (**figure 4-6**). In exploring proteins individually, the literature is found to be rich with studies correlating some if not all with malignancy as well. So, it is justifiable to write a brief description of each protein with a great emphasis on the selected ones and the reason behind selecting them.

The first protein is Actin cytoplasmic 2 or Actin γ 1 (42 kDa) which is one of the most condensed forms of protein ubiquitously expressed in all eukaryotic cells. It is a key cytoskeletal protein involved in various types of cell motility, and maintenance of the cytoskeleton. Previous studies have described the presence of the other isoforms of Actin (Alpha and Beta). However, actin, beta and gamma co-exist in most cell types thus comparison was done across the studies (**table 4-9**). De Jong et al., (2010) identified Actin as a promising salivary biomarker in differentiating pre- malignant and malignant oral lesions and has supported their results by a number of studies. In the present study patients who are at risk of developing MALT-L were compared to SS patients. There was a discrepancy between each run where in the first run there was a of 2.4 fold change ratio in SS at risk of MALT-L development compared to SS sample while in the second run was 1 (**table 4-2**), thus this protein was excluded despite its great potential. In further work, it may be appropriate to investigate actin levels in further patients at risk of developing MALT-L.

The second protein is Ig γ -1 chain C region or IgG1 (36 kDa) which is the constant region of one of the four subclasses of immunoglobulin (Ig). It is the most abundant class of immunoglobulin found in the plasma but only 5-15% of salivary Ig is made up of mainly IgG and IgM which are to some extent, derived from gingival crevicular fluid (GCF) or from plasma leakage (Chiappin et al., 2007). However, >70% of IgG in saliva is thought to be of local origin (Edgar et al., 2004). Other than the aforementioned studies (**table 4-9**), a study by Donadio et al., (2013) has reported an up-regulation of IgG and Protein S100-A9 in benign tumour of parotid glands linking it to autoimmunity and inflammation, thus supporting our findings. Also,

it was attributed to B cell activation in periphery according to Katsiogiannis and Wong, (2016). However, the fold change ratio (1.004) was below the cut- off when SS at risk of MALT-L was compared to SS sample in the second run as shown in **table 4-2** and it was omitted as well.

The last two proteins which are pro-inflammatory members of the Alarmin family, they are myeloid-related, calcium binding proteins; S100-A9 (Calgranulin B) (11 kDa) and S100-A8 (Calgranulin A) (13 kDa) which are predominantly found as S100A8/A9 heterodimer having a broad range of intracellular and extracellular functions; e.g. playing a prominent role in the regulation of inflammation (chronic and acute) inducing leukocyte chemotaxis and adhesion. Originally, S100A8/A9 was discovered as an immunogenic protein expressed by neutrophils with potent anti-microbial properties referred to by its former name calprotectin in 1990 (Fagerhol et al., 1990). It has been reported as being up-regulated in many cancers including melanoma, gastric cancer, breast cancer and colorectal cancer (Bresnick et al., 2015, Salama et al., 2008). Furthermore, changes in S100A9 protein expression levels are known in literature to be linked with autoimmune and inflammatory diseases such as arthritis, rheumatoid arthritis, systemic sclerosis and systemic lupus erythematosus (Foell and Roth, 2004).

S100A8 and S100A9 were differently expressed in SS patients in the present study as well as other studies as shown in **table 4-9**.

Both proteins were increased in diseased samples when compared to controls in both runs and the fold change ratio was 2.4 and 9.6 for S100A8 while it was 2.1 and 12.1 for S100A9 in the first and second runs respectively, when samples of patients at risk of developing lymphoma were compared to SS samples seen in **table 4-2**. Therefore, it was decided to choose the heterodimer of both for further assessment via ELISA.

4.5.2 Preclinical validation of selected protein

Can one of the candidate proteins identified in the proteomics study be selected for a large cohort of subjects and might it be able to discriminate between different groups of SS subjects? Moreover, are there associations between the candidate proteins and the overall impairment of salivary gland function?

Immunoassays, such ELISA or western blotting, are the most frequently used methods for validation of saliva biomarkers and if properly used, ELISA is a sensitive, accurate and rapid detection method. It is especially effective when large numbers of samples must be assayed thus it was selected.

4.5.2.1 Salivary S100A8/A9 levels and their associations

The ELISA analysis indeed supported the proteomics findings, showing similar expression profile trends being higher in parotid saliva of SS groups compared to healthy controls in addition to the newly added disease controls (**figure 4-12**). Although the levels in whole mouth saliva were higher (6.8 times) than parotid saliva for SS patients which might be expected as it is released from other sources, but they were not high enough to be significantly different than the healthy and disease controls (**figure 4-14**) (whole saliva is 38 times higher in healthy controls and 29 higher in disease controls than parotid saliva).

A possible explanation is that despite being strict in selecting age- matched healthy controls who were not on any xerostomic medication, it might be that influences on oral health status (e.g. caries, periodontal disease, fungal infection and ulcers) affected S100A8/A9 levels in whole mouth saliva. Furthermore, it is extremely difficult to collect a true unstimulated whole saliva sample without oral musculature movement (shedding cells) which may contribute to the higher readings. Also, it is worth noting that almost half of the SS subjects didn't produce whole mouth saliva but if collection had been possible the assayed values might have been different.

In general, our results were comparable to previously published work by Sweet et al., (2001), reporting higher levels of S100A8/A9 (calprotectin) in whole saliva of SS patients in comparison to healthy controls. Our results showed higher values but not significant enough as explained previously. Interestingly, Sweet and colleagues have reported no correlation between parotid and whole saliva levels attributed to not being of a salivary gland origin and this is supporting our findings regarding the healthy control group, where no correlation was found between parotid and whole saliva levels. On the other hand, it contrasted with findings in SS patients suggesting that it might be of salivary gland origin or neighbouring tissue since they are the major site of inflammation in this group (weak correlation).

It is important to note that there was a wide range of calprotectin levels (200-18000 ng/mL) in whole saliva reported by this group (Sweet et al., 2001). It is similar to our findings but the parotid saliva levels that they found in SS patients were not found. Levels in parotid saliva of healthy controls were comparable to ours and to another study by Muller et al., (1993) where they compared parotid saliva levels of calprotectin in controls and HIV patients. They have reported that it might be derived from squamous epithelial cells lining the large parotid ducts also from stromal granulocyte and macrophages.

Cuida and co-workers studied calprotectin levels in LSG of SS patients, as well as for the salivary levels (stimulated whole mouth saliva) in SS and healthy subjects, they reported no differences between control and SS levels (Cuida et al., 1993). Their other study reported a positive correlation between plasma and whole mouth levels of SS patients despite being higher in the latter. Also, there was a weak negative correlation with the flow rate and this was comparable to our parotid saliva results where a weak negative correlation was found between parotid flow rate and salivary parotid levels whereas our whole mouth results showed no correlation between flows and S100A8/A9 levels (Brun et al., 1994).

Furthermore, they analysed levels of calprotectin in different oral fluids of healthy subjects. They have showed greater levels in whole mouth saliva than parotid saliva and mucosal transudate had even higher levels than both types of saliva. Their reported parotid values were

ten- fold higher than our findings and the aforementioned studies which could be due to different collection methods since we stimulated the flow while they analyse unstimulated saliva which can have higher protein concentrations (Cuida et al., 1995). In their later study they have added the comparison of parotid and whole saliva levels in SS patients and controls and reported a positive correlation between parotid levels and the focus score of SS patients (Cuida et al., 1997). Furthermore, it should be noted that in the present study none of the flow rates correlated with USS. Salivary levels of the above-mentioned studies are summarised in **table 4-10** together with our results.

Subgrouping of SS has revealed significant differences in parotid S100A8/A9 levels of SS patients who developed and are at risk of developing MALT-L in comparison to healthy controls (**figure 4-12**). While S100A8/A9 levels in whole mouth saliva were significantly elevated in SS patients who developed lymphoma when compared to both disease and healthy controls as well as to SS group (**figure 4-14**). This confirms the important link of S100A8/A9 to cancer as an amplifier of not only inflammation but associated to tumour development which has been previously reported (Salama et al., 2008, Bresnick et al., 2015).

Interestingly it was noted that when comparing left and right glands of 2 patients at risk of developing MALT-L, that the swollen glands showed increased level of S100A8/A9 compared to the contralateral unswollen gland.

Table 4-10 Summary of salivary levels of calprotectin in literature

Study references	saliva	Mean CT levels(ng/mL)	Mean SS levels(ng/mL)	Significant
Muller 1992	sParotid	207 *	-	-
Cuida et al.1993	sWhole	27100	29000	No
Brun et al.1994	sWhole	27100	23624	No
Cuida et al. 1995	uParotid	3200	-	-
	sWhole	22000	-	-
Cuida et al. 1997	sParotid	2700	5500	No
	sWhole	15200	36300	No
Sweet et al. 2001	sParotid	300	Not written	Not found
	uWhole	2000	14000	Yes
our results	sParotid	251.4	1968.8	Yes
	uWhole	9545.2	13401	No

*median, uWhole; unstimulated whole saliva, sWhole; stimulated whole saliva, sParotid; stimulated parotid saliva, uParotid; unstimulated parotid saliva.

4.5.2.2 Receiver operator characteristic (ROC) curve

The diagnostic accuracy of the S100A8/A9 ELISA test in differentiating SS group from the control subjects has identified rather a fair cut-off levels in both whole and parotid saliva demonstrating better accuracy of parotid saliva vs. whole saliva; 82 % sensitivity vs. 73% and 56% specificity vs. 50%.

While a good diagnostic accuracy was found in differentiating the SS subgroups via whole saliva levels which could differentiate between SS with developed MALT-L and controls as well as SS subgroup having slightly higher sensitivity and specificity when compared to controls (85% sensitivity and 80% specificity).

These readings (cut-offs) might be of significance since the sensitivity and the reproducibility of the actual test (ELISA) was further done demonstrating an acceptable recovery readings.

4.5.2.3 Salivary flow rates and the total protein concentration

A diminished mean flow of WFR was evident in the overall SS patients particularly those who are at risk or already developed MALT-L (**figure 4-8**), specifically where almost half of the patients could not provide a sample of WMS, evidence from the literature was mentioned previously in chapter 3. On the other hand parotid saliva flow rates did not differ between control groups and the overall SS group as well as the SS subgroup (**figure 4-9**), and again this has been reported by others (Fox et al., 1987, Thorn et al., 1989, Skopouli et al., 1989, Atkinson et al., 1990, Atkinson, 1993, Carpenter et al., 2000, van den Berg et al., 2007) but not by other studies such as Stuchell et al., (1984), Kalk et al. (2001a), (2002c) and Pedersen et al., (2005). Although the underlying mechanism is not yet understood, a possible explanation for it is the involvement of the submandibular and sublingual glands in SS at first as mentioned previously. However, lymphoma patients did have lower parotid flows compared to the controls and patients at risk of MALT-L had lower but not statistically significant, confirming the involvement of parotid glands at this later stage of SS.

It is worth noting that the original cohort of parotid samples was even larger and included mucous like secretions (mucus plugs), obtained mostly from the SS patients who were at risk or had already developed lymphoma and in some SS patients with longer disease duration. These samples were excluded since the mucus interfered with ELISA. Furthermore, in chapter 3 a significant difference was found between overall SS patients and disease control patients and this is largely due to the inclusion of more patients in the previous study especially at risk patients and those who developed MALT-L.

Regarding the total protein concentration it was noted that there was higher values in whole mouth saliva of SS compared to controls (**figure 4-10**) which is supported by other studies (Fox et al., 1987, van der Reijden et al., 1996, Thorn et al., 1989, Hernandez-Molina et al., 2011). On the other hand, parotid total protein concentration did not change between the groups (**figure 4-11**), which is in agreement with the findings of others (Carpenter et al., 2000, Kalk et al., 2001a) but contrast with Hu et al., (2007a) who found a higher concentrations in SS group. A negative correlation was observed between flow and total protein concentration of whole saliva, while the parotid parameters did not correlate (**table 4-4**), in agreement with Carpenter et al., (2000).

In conclusion, this study supports previous reports indicating the value of saliva as a diagnostic tool of disease activity and progression in Sjögren's syndrome. The preliminary results of parotid saliva proteomic analysis indicate a list of candidate markers of SS and SS at risk of lymphoma. Salivary levels of S100A8/A9 tend to reflect more local inflammatory activity showing the greatest difference between groups in parotid saliva. Furthermore, the changes in sialochemical analysis (total protein concentration) and sialometric analysis (flow rate) seen with SS patients were indicated by previous studies. Further studies are needed to better clarify the relationship of salivary S100A8/A9 with the focus score and possibly tear fluid to better understand SS glandular disease activity. Analyses of S100-A8 and -A9 individually would also be of great of interest.

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5 Salivary cytokines in Sjögren's syndrome

5.1 Introduction

Cytokines are small, soluble peptides or proteins that are produced by a variety of cells that participate in the immune response, including T cells, B cells, macrophages and others. They are powerful immune regulators constituting the communication network of the immune system. Any alteration in cytokine expression may lead to inflammation, allergy, immune deficiency or autoimmunity. Exocrine glands are assumed to be the target of lymphocytic hyperactivity in primary SS. Therefore, we found it of significance to study the salivary glands' secretions to better understand the disease activity. Saliva in particular would be of interest, since collection of this body fluid is a non-invasive procedure. Cytokine environment provides important visions into pathological processes and can potentially serve as biomarkers for Sjögren's syndrome (Zhang and An, 2007, Roescher et al., 2009). Furthermore they play important roles in B-cell activation, proliferation and apoptosis and thus may be etiologically related to risk of B-cell non-Hodgkin lymphoma (Dong et al., 2013). (See chapter one subsection 1.4.2.2 and 1.6.3.1).

Several investigators have measured specific cytokine concentrations in the saliva in SS showing that alterations in salivary cytokine profiles may be useful for both its diagnosis and progression (Ohyama et al., 2015, Moriyama et al., 2012, Hernandez-Molina et al., 2011, Fox et al., 1994, Kang et al., 2011, Rhodus et al., 1998, Nguyen et al., 2008). Therefore, in this chapter we have focused on cytokine levels in saliva and reviewed the literature regarding cytokines in some of the other biological samples of SS patients in general.

Due to the heterogeneous nature of SS, it has been suggested that no single marker is likely to be sufficient for the prediction of a disease state and a panel of biomarkers should be identified. This could be accomplished by utilizing proteomic approaches on saliva samples that have the capacity of profiling multiple biomarkers. While cytokines detection was challenging via the aforementioned proteomic analysis (described in chapter 4) owing to their low abundance, the proteome profiler human cytokine array test (membrane-based sandwich

immunoassay) and luminex multiplexed assay (bead-based assays) provided the alternative solutions; enabling the identification of the relative expression of multiple cytokines in a single test. These antibody- based methods allow greater sensitivity of detection. Cytokine antibody arrays that bind and detect proteins from samples (semi-quantitatively) and multiplex antibody based assay using bead- based systems that allow a more sensitive simultaneous quantification of multiple proteins. These are particularly useful for cytokines and growth factors which are low abundant proteins.

In this study, cytokine profiles of parotid samples from healthy, SS, SS at risk and those who already developed lymphoma were explored via a cytokine antibody array. Bead- based assays were then used for measuring the levels of selected cytokines in a larger group of subjects with the addition of disease control patients (SNOX). Furthermore, the list of cytokines was narrowed and measured via more sensitive performance bead-based assays; where whole mouth saliva was included as well and compared.

5.2 Aims and objectives

Given the important role of cytokines in Sjögren's syndrome (SS), the hypothesis behind the study described in this chapter was:

Cytokines are present in saliva and can differentiate between SS, healthy controls and other forms of dryness (SNOX), further they can distinguish different subgroups of SS. Thus, the aims were as follow:

1. Identify parotid salivary cytokine biomarkers for SS using a cytokine antibody array approach to determine whether they are involved in SS, SS at risk or already developed lymphoma via comparison to healthy control samples.
2. Use the selected cytokines and compare their levels in SS to healthy and disease controls (SNOX) via bead- based multiplex antibody assays.
3. Compare their levels in SS groups of different subgroups; SS, SS at risk and those who already developed lymphoma via bead- based assay.

Also in this study, the group of selected cytokines were further narrowed and tested via more sensitive assays and the specific aims were under taken:

1. Verify the above-mentioned aims to identify saliva indicators which are associated with the different subgroups of SS.
2. Assess whole mouth salivary cytokines in relation to the above-mentioned groups of patients and compare to levels in parotid saliva.
3. Establish whether there is an association between the candidate cytokines with the overall salivary gland function.

5.3 Materials and methods

5.3.1 Study group

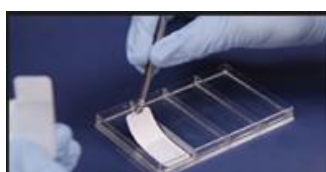
Twelve age and sex matched subjects from 4 groups (healthy controls (n=3), SS (n=3), SS at risk of developing MALT-L (n=3) and SS who already developed it (n=3)) were included in the proteome profiler analysis.

For further analysis via multiplex immunobead- assays (Luminex, R & D Systems, Minneapolis, MN), samples were included to a total number of 76 for initial screening assays then incorporated additional samples reaching to 82 samples for performance assay and 57 whole mouth saliva samples. For both assays a fifth group was added which is the disease control (SNOX) group.

5.3.2 Saliva sample collection

Stimulated parotid saliva samples (n=76 for screening) and (n=82 for performance) were collected and stored as outlined in chapter 2, subsection 2.1.4 and **Appendix 4& 5**. Unstimulated whole mouth saliva samples were collected as well (n=57) and analysed using the performance assay only. All samples were kept on ice and stored until required. Their flows and total protein concentration were measured as described in chapter 2, subsection 2.2.1.

Human XL Cytokine array analysis
(Proteome profiler)
102 analytes



The Bio-Plex® 200 system
Luminex® 100/200™ instrument



Magnetic Performance
Luminex Assay
6 analytes



Magnetic Screening
Luminex Assay
20 analytes

Figure 5-1 Schematic presentation of the work flow for cytokine analyses

Involving proteome profiler followed with 2 types of Luminex assays (screening then performance) (R and D systems, Minneapolis MN).

5.3.3 Laboratory analyses

5.3.3.1 Proteome Profiler™ Human XL Cytokine Array

Three sets (kits) of parotid saliva samples (4 samples each) were analysed; their cytokine levels were determined semi-quantitatively with a multi-cytokine (n=102) membrane array (R & D Systems, Minneapolis, MN). The kit (cat. no. ARY022) was used according to manufacturer's instructions (further details are found in chapter 2 subsection 2.2.6 and **Appendix 7**).

5.3.3.2 Luminex® xMAP Assays

5.3.3.2.1 Magnetic Screening Luminex Assay

Two multiplex bead- based kits (R&D Systems, Minneapolis, MN) were used to measure the concentrations of a total of 20 cytokines in parotid saliva. The first kit (cat. no. LXSAHM-3) was used to measure IL-3, and ST2. While the second kit (cat. no. LXSAHM-19) measured IFN- γ , TNF- α , IL-1 α , -1 β , -2, -4, -5, -6, -10, -12p70, -17A, -22, -23 and -33) and chemokines (MCP-1, MCP-3, IP10 and IL8). Samples were thawed directly on the day of analysis and the assays were performed as previously described (chapter 2 subsection 2.2.7.1 and **Appendix 8**).

5.3.3.2.2 Magnetic Performance Luminex Assay

Commercial customised panel (R&D Systems, Minneapolis, MN) (cat. no. FCSTM03) was used to measure the levels of selected six cytokines (IL-1 α , -1 β , -4, -6, -8 and MCP1) in parotid and whole mouth saliva samples, using a similar protocol to the screening assay. (Refer to chapter 2 subsection 2.2.7.2 and **Appendix 8**).

5.3.4 Statistical analysis

All statistics were evaluated using SPSS, and GraphPad Prism® 6 (GraphPad Software Inc., California, USA) was used for the graphical presentation, where all the Data were transferred to Windows® Excel 2007 spread sheet.

5.4 Results

5.4.1 Proteome Profiler™ Human XL Cytokine Array

5.4.1.1 Study group

Table 5-1 Characteristics of patients included in the proteome profiler

Groups	Controls	SS	SS at risk	SS/ MALT-L
Age	51.7±7	53.3±7.4	50±8.6	52.7±11.8
Gender	F	F	F	F
Count	3	3	3	3

The mean densities of spots on the membranes were quantified by ImageJ software, an example of one kit with 4 membranes is shown in (figure 5-2) and difference between the respective cytokine levels is represented as fold change and p-values which is seen in later graphs and tables.

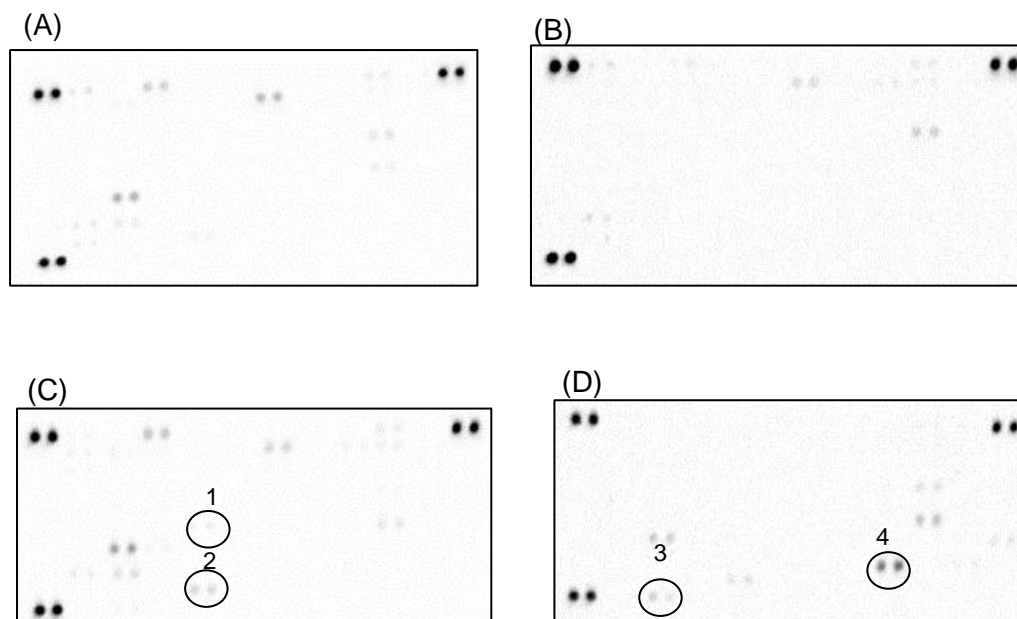


Figure 5-2 Represents an example of one sample per membrane of cytokine antibody array (n=4)

(A) Healthy control

(B) Sjögren's syndrome (SS)

(C) SS at risk of developing mucosa associated lymphoid tissue lymphoma (MALT-L)

(D) SS developed MALT-L.

Examples of over-expressed cytokines/proteins are seen; (1) IL-10, (2) trefoil factor 3 (TFF3), (3) Vitamin D Binding protein, (4) TNF-α.

Based on the cytokine array profiling, cytokines (proteins) were ranked at first according to their relative expression in samples; first ranked proteins were defined as being identified in all 12 samples (45/102 cytokines); second ranked proteins were identified in 11 samples (37/102) and the third ranked proteins were identified in 10 or less samples (20/102).

Then these three ranks were classified individually by fold change with an arbitrary cut-off of 1.5, starting with comparisons between control samples to all of the disease samples (SS, SS at risk and those with MALT-L). For the first ranked proteins, 3 were up-regulated when SS was compared to healthy while 4 were down-regulated. Eight proteins were up-regulated when SS at risk was compared to control samples. Twenty-one proteins were up-regulated when SS with MALT-L was compared to the healthy control group.

Comparisons of SS to the rest of the disease samples have revealed; 14 up-regulated proteins when SS at risk was compared to SS while one was down-regulated. Twenty-six proteins were up-regulated when patients with MALT-L were compared to SS and one was down-regulated. Fourteen proteins were up-regulated, when SS patients with MALT-L were compared to those who are at risk of developing it.

For the second ranked proteins, in SS to controls comparisons there were 2 up-regulated proteins and 5 down-regulated. There were 13 and 19 up-regulated proteins when SS at risk and SS who already developed the cancer were compared to controls respectively. Twenty-eight proteins were up-regulated when SS at risk and confirmed MALT-L were compared to SS group each. Nine up-regulated while 3 down-regulated proteins were identified when the SS group with MALT-L was compared to the SS at risk group.

In the third ranked proteins, 5 proteins were down-regulated when SS was compared to control subjects while 3 and 7 up-regulated proteins were identified when SS at risk and SS with MALT-L were compared to controls correspondingly. Comparisons of SS at risk and SS with MALT-L to SS group have identified 16 and 14 up-regulated proteins respectively, while none were found when SS with lymphoma was compared to SS at risk group.

To increase the stringency of results, these proteins were further ranked by p-value (one- way ANOVA followed by Tukey HSD post hoc test); 2 proteins (ST2 and vitamin D BP) and 3 proteins (ST2, Endoglin and IL-22) were significantly different when SS at risk was compared to control and SS groups individually, while one protein (CD30) was statistically different when SS with MALT-L was compared to SS patients from the first ranked proteins.

From the second ranked proteins; one protein (MCP-1) was significantly different when comparison between SS at risk and control groups was done, while 4 proteins (MCP-1, -3, IL-10 and IL-32 $\alpha/\beta/\gamma$) were different when SS at risk was compared to SS patients. None of the third ranked proteins were statistically different between the groups. Summary of the results is found in (**figure 5-3**).

An example of the first ranked (45) cytokines/proteins ordered by fold change and p-value is shown in **tables 5-2 & 5-3** when compared to controls then when compared to SS. The second and third protein summaries are found in **Appendix 14 to 17**.

Data were plotted as fold change (\log_2 fold change ratio) and significance ($-\log_{10}$ p-value) in a volcano plot for the first and second rank proteins (**figures 5-4 and 5-5**) while the third rank proteins were not plotted as none showed any significant differences.

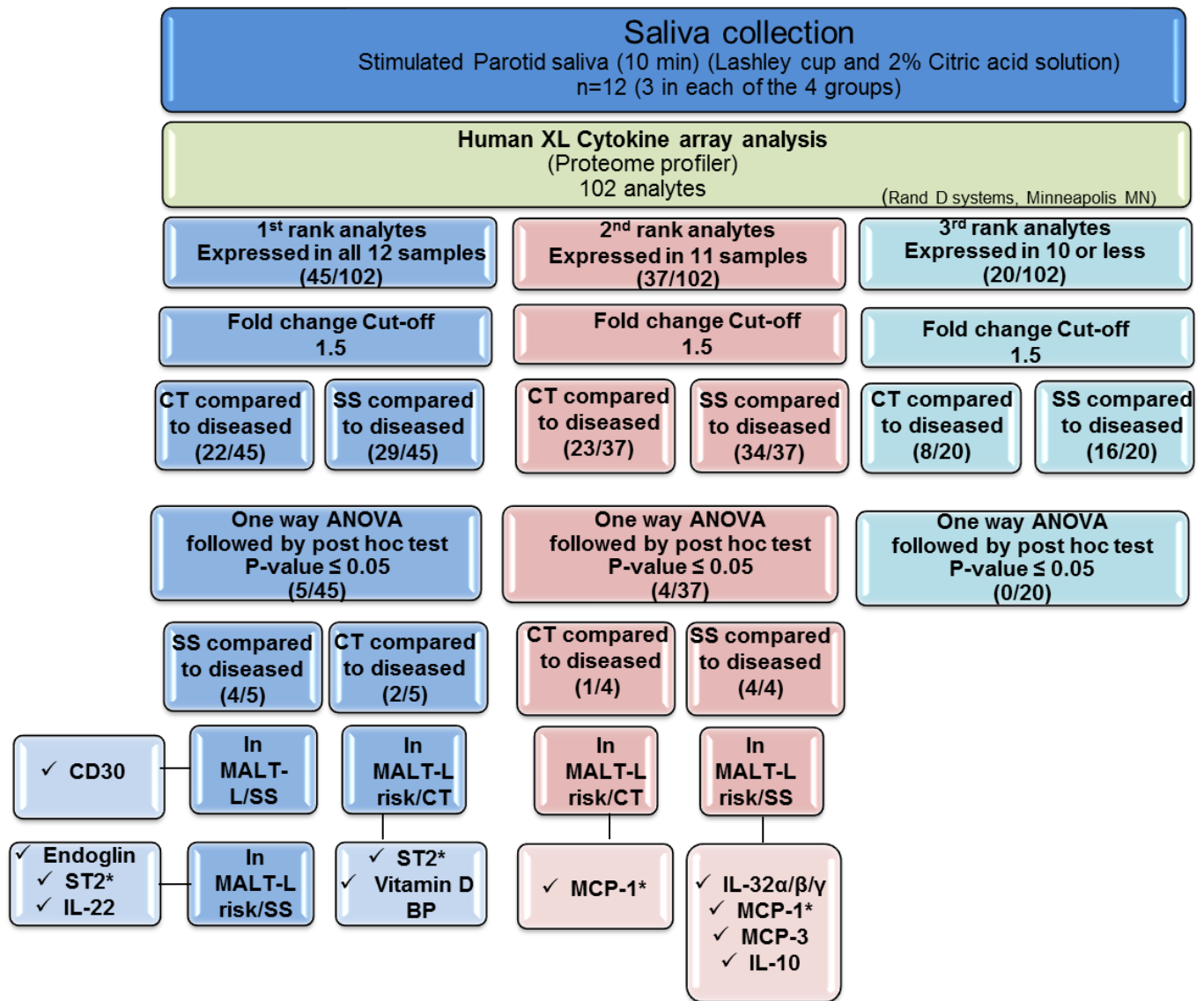


Figure 5-3 Work flow of the different type analyses generated on data obtained from the proteome profiler. CT; healthy controls, SS; Sjögren's syndrome, MALT-L risk; SS at risk of developing MALT-L, MALT-L; mucosa associated lymphoid tissue lymphoma. * a common protein.

CT compared to disease which are SS, SS at risk and with MALT-L.

SS compared to disease which are SS at risk and with MALT-L.

Table 5-2 Summary of the first rank cytokines-proteins ordered by fold change and p-value when compared to controls

		SS vs. CT		MALT-L risk vs. CT		MALT-L vs. CT
Analytes (22/45)	Fold change	p- value	Fold change	p- value	Fold change	p- value
(A) MIG	1.9	NS	1.6	NS	5.5	NS
(B) ST2	>-1.5, <1.5	NS	1.8	0.005*	1.6	NS
VEGF	>-1.5, <1.5	NS	2	NS	3.9	NS
Resistin	>-1.5, <1.5	NS	1.9	NS	6.4	NS
MMP-9p	>-1.5, <1.5	NS	1.8	NS	5.6	NS
IL-18 Bpa	>-1.5, <1.5	NS	1.6	NS	2.9	NS
IL-33	>-1.5, <1.5	NS	1.6	NS	1.7	NS
IP-10	3.7	NS	>-1.5, <1.5	NS	4.7	NS
IL-16	2	NS	>-1.5, <1.5	NS	2.5	NS
(C) Vitamin D BP	>-1.5, <1.5	NS	>-1.5, <1.5	0.04*	4.6	NS
RBP4	>-1.5, <1.5	NS	>-1.5, <1.5	NS	5.4	NS
uPAR	>-1.5, <1.5	NS	>-1.5, <1.5	NS	3.7	NS
IL-19	>-1.5, <1.5	NS	>-1.5, <1.5	NS	3.5	NS
ENA-78	>-1.5, <1.5	NS	>-1.5, <1.5	NS	3.4	NS
Endoglin	>-1.5, <1.5	NS	>-1.5, <1.5	NS	2.4	NS
GDF-15	>-1.5, <1.5	NS	>-1.5, <1.5	NS	2.1	NS
Fas Ligand	>-1.5, <1.5	NS	>-1.5, <1.5	NS	1.7	NS
Complement factor D	-1.9	NS	>-1.5, <1.5	NS	1.6	NS
HGF	-2.2	NS	>-1.5, <1.5	NS	1.6	NS
EMMPRIN	-1.5	NS	>-1.5, <1.5	NS	1.5	NS
IL-4	-3.3	NS	>-1.5, <1.5	NS	1.6	NS
Serpin	>-1.5, <1.5	NS	1.9	NS	>-1.5, <1.5	NS

Fold change in the first group (A) is ≥ 1.5 in all disease groups. In the second group (B) it is increased in 2 of the disease groups. While the third group (C) fold change is increased in one group only. NS; not significant, CT; healthy controls, SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma. IL-1 receptor-like 1(ST2) and Vitamin D BP were significantly different when compared to controls (one- way ANOVA followed by Tukey HSD post hoc test). Blue up-regulated red down- regulated.

First ranked proteins were defined as being identified in all 12 samples

Table 5-3 Summary of the first rank cytokines-proteins ordered by fold change and p-value when compared to SS

		MALT-L risk vs. SS		MALT-L vs. SS		MALT-L vs. at risk	
Analytes (29/45)	Fold change	p- value	Fold change	p- value	Fold change	p- value	
(A)							
ENA-78	1.5	NS	4	NS	2.6	NS	
GDF-15	1.6	NS	3.4	NS	1.8	NS	
IL-19	2.3	NS	4.8	NS	2.6	NS	
Endoglin	1.6	0.02*	3.3	NS	1.7	NS	
(B)							
IL-4	3.5	NS	4.9	NS	>-1.5, <1.5	NS	
Fas Ligand	2	NS	3.1	NS	>-1.5, <1.5	NS	
HGF	1.6	NS	2.6	NS	>-1.5, <1.5	NS	
IL-33	2.5	NS	2.6	NS	>-1.5, <1.5	NS	
IL-22	3.5	0.03*	2.8	NS	>-1.5, <1.5	NS	
ST2	2	0.007*	1.9	NS	>-1.5, <1.5	NS	
IL-6	2.3	NS	2.2	NS	>-1.5, <1.5	NS	
IL-11	2	NS	1.5	NS	>-1.5, <1.5	NS	
TfR	2.5	NS	2.4	NS	>-1.5, <1.5	NS	
EMMPRIN	>-1.5, <1.5	NS	3	NS	1.6	NS	
Resistin	>-1.5, <1.5	NS	5.8	NS	4.4	NS	
RBP4	>-1.5, <1.5	NS	4.6	NS	4.5	NS	
uPAR	>-1.5, <1.5	NS	4.6	NS	3.4	NS	
MMP-9	>-1.5, <1.5	NS	4.2	NS	3.8	NS	
Vitamin D BP	>-1.5, <1.5	NS	4.1	NS	3.4	NS	
MIG	>-1.5, <1.5	NS	3.6	NS	3.9	NS	
VEGF	>-1.5, <1.5	NS	3	NS	1.9	NS	
(C)							
CD40 ligand	1.5 }	NS	>-1.5, <1.5	NS	>-1.5, <1.5	NS	
CD30	>-1.5, <1.5	NS	2	0.05*	>-1.5, <1.5	NS	
IL-18 Bpa	>-1.5, <1.5	NS	2.7		>-1.5, <1.5	NS	
Complement factor D	>-1.5, <1.5	NS	2.6		>-1.5, <1.5	NS	
Thrombospondin-1	>-1.5, <1.5	NS	2		>-1.5, <1.5	NS	
GM-CSF	>-1.5, <1.5	NS	1.5	NS	>-1.5, <1.5	NS	
IL-16	>-1.5, <1.5	NS	-1.7	NS	1.5 }	NS	
IP-10	-2.8	NS	>-1.5, <1.5	NS	3.8 }	NS	

Fold change in the first group (A) is ≥ 1.5 in all disease groups. In the second group (B) it is increased in 2 of the disease groups. While the third group (C) fold change is increased in one group only. NS; not significant, CT; healthy controls, SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma. Endoglin, IL-1 receptor-like 1(ST2), CD30 and IL-22 were significantly different when compared to SS (one- way ANOVA followed by Tukey HSD post hoc test). Blue up-regulated red down- regulated. First ranked proteins were defined as being identified in all 12 samples

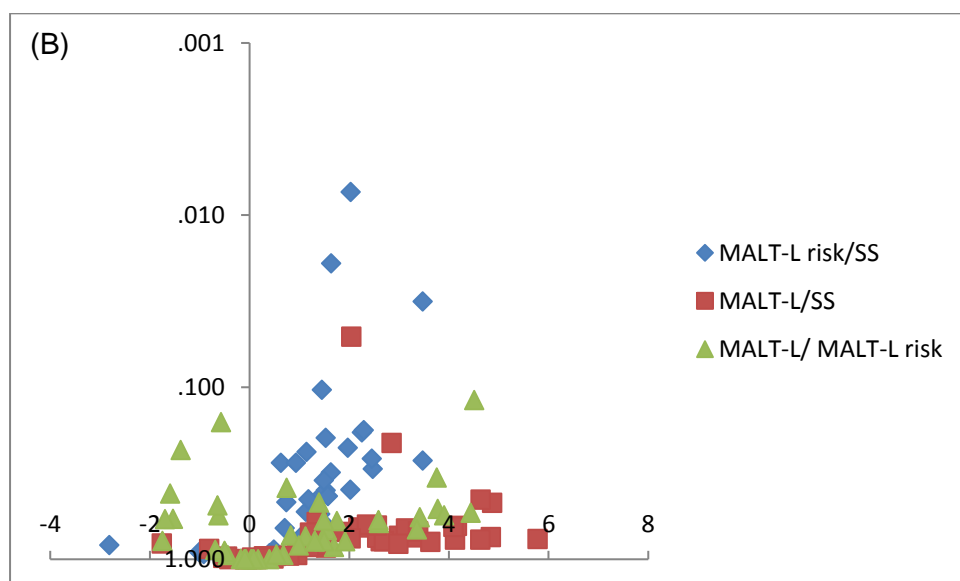
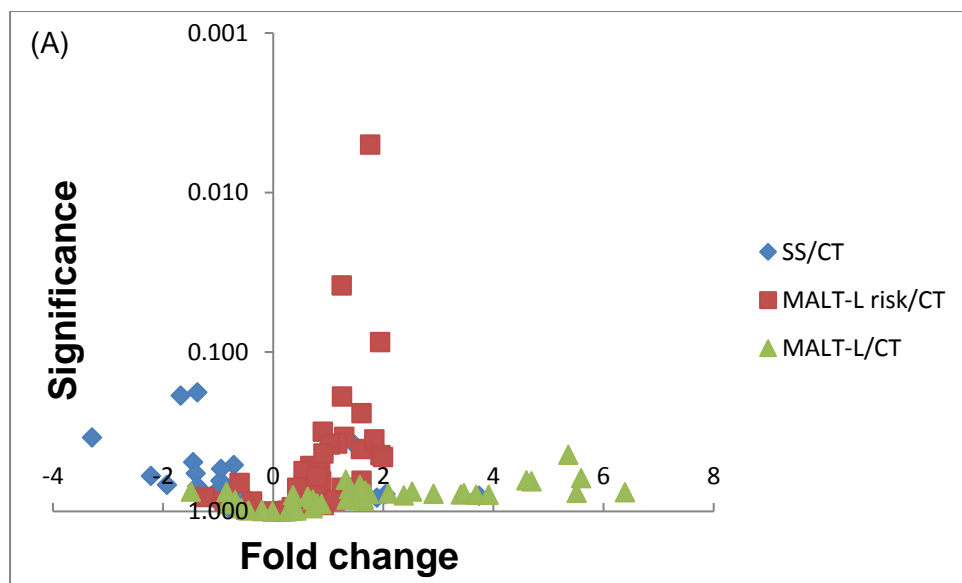


Figure 5-4 Volcano plot representing the significance ($-\log_{10}(\text{p-value})$) and the fold change of the first rank cytokines/proteins (45/102) identified in all diseased groups when compared to controls (A) and to SS (B).

CT; healthy controls, SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma.

First ranked proteins were defined as being identified in all 12 samples

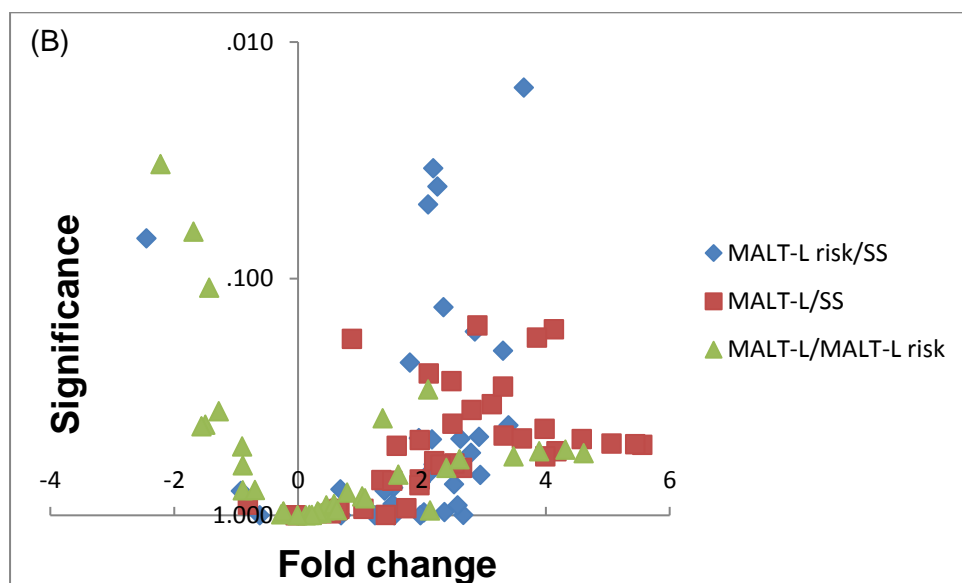
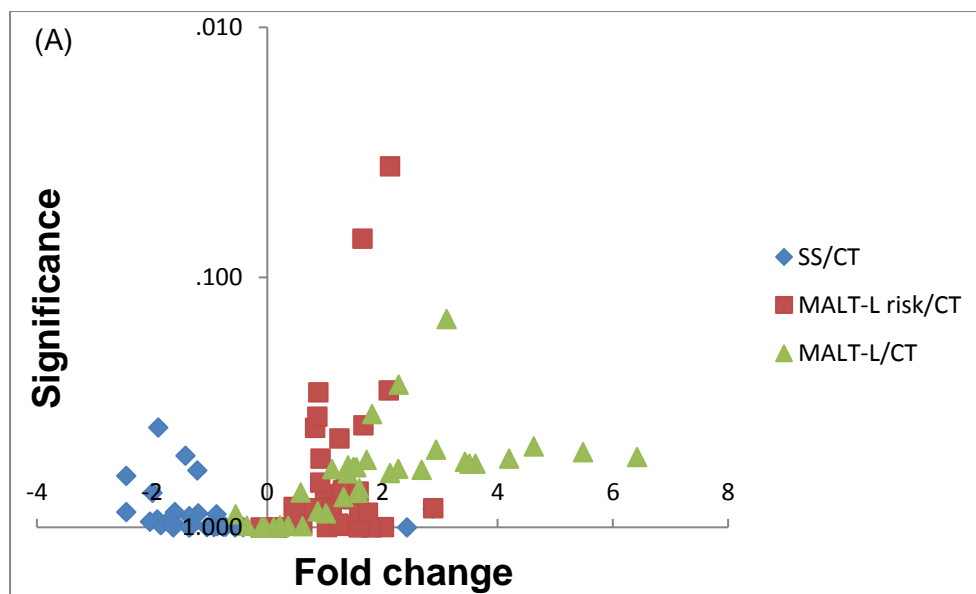


Figure 5-5 Volcano plot representing the significance ($-\log_{10}(\text{p-value})$) and the fold change of the second rank cytokines/proteins (37/102) identified in all diseased groups when compared to controls (A) and to SS (B).

CT; healthy controls, SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma.

Second ranked proteins were identified in 11 samples of 12

5.4.2 Luminex Screening Immunobead assay

5.4.2.1 Study group

Seventy- six subjects were included for this study and their parotid samples were analyzed, demographics and diagnosis break down are summarised in table 5-4. Salivary levels of 20 cytokines were compared among SS patients and control subjects. The cytokines (n=20) assayed included: IFN- γ , TNF- α , IL-1 α , -1 β , -2, -3, -4, -5, -6, -8, -10, -12p70, -17A, -22, -23, -33, IP10, MCP-1, MCP-3 and ST2.

Table 5-4 Demographic and clinical characteristics of the SS patients and control subjects included in Luminex screening assays

Groups	Age	sex	SPFR (ml/min)
CT (n=17)	50.2 \pm 2.9	1M 16F	0.34 \pm 0.04
SNOX (n=10)	63.4 \pm 1.8	0M 10F	0.34 \pm 0.09
Overall SS (n=49)	52.5 \pm 1.7	1M 48F	0.28 \pm 0.03
SS (n=20)	51.5 \pm 2.7	1M 19F	0.36 \pm 0.06
SS at risk (n=18)	51.9 \pm 3.01	0M 18F	0.26 \pm 0.05
SS/ MALT-L (n=11)	55.2 \pm 3.2	0M 11F	0.15 \pm 0.05*

CT; healthy controls, SNOX; sialadenitis, nodular osteoarthritis and xerostomia, SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, SPFR; stimulated parotid flow rate. Data are reported as mean \pm SEM. * SS/MALT-L is significantly reduced compared to healthy controls (Kruskal-Wallis test followed by Dunn's post hoc test).

5.4.2.2 Cytokine levels:

Values for cytokines in many samples were too low and outside of the standard curve. Out of the 20 cytokines, 3 were not detectable in parotid saliva of all the subjects; IL-5, -10, and -17, while IL-12p70 was detected in only 2 patients (SS and SS at risk) whereas it was not detected in the rest of subjects. Few were not detected in some subgroups. On the other hand, levels of IP10 in all the samples exceeded the dynamic range and required further dilution. Levels of IL-8 and MCP-1 in few patients were above the detection limit which also needed further dilution.

IL-1 α level was found to be statistically higher in the overall SS group compared to the diseased control group ($p=0.002$). Subgrouping revealed increased levels of IL-1 α in SS patients at risk and those who already developed lymphoma compared to SNOX patients ($p=0.046$ and 0.003 respectively) (**figure 5-6**). Increases in IL-4, -6 and MCP-1 levels in overall SS group relative to SNOX group was identified ($p=0.018$, >0.0001 and 0.011). IL-6 was statistically higher in overall SS group in comparison to healthy controls as well ($p=0.009$). Interestingly levels of MCP-1 were lower in SNOX patients compared to healthy controls ($p=0.027$). While IL-8 was not significantly different in overall SS group compared to controls (**figures 5-6 and 5-7**).

Furthermore, levels of MCP-1 were higher ($p=0.024$) in SS patients who developed MALT-L relative to SNOX group while levels of IL-8 were higher in SS patients at risk of developing lymphoma compared to SNOX ($P=0.012$) and even SS group ($p=0.009$) when SS patients were subgrouped. Levels of IL-6 was elevated in the MALT-L group compared to healthy ($p=0.028$) and disease ($p=0.001$) controls. While the levels in SS and MALT-L risk groups were higher than the disease control group ($p=0.024$ and 0.004 correspondingly). On the other hand, IL-4 levels were high in the MALT-L group but it was not significant ($p=0.051$) (**figures 5-6 and 5-7**). The other cytokines showed no significant differences between groups. Moreover, almost all positive samples were very close to the low sensitivity point of the kit. Salivary levels of all the cytokines are summarised in **table 5-5**.

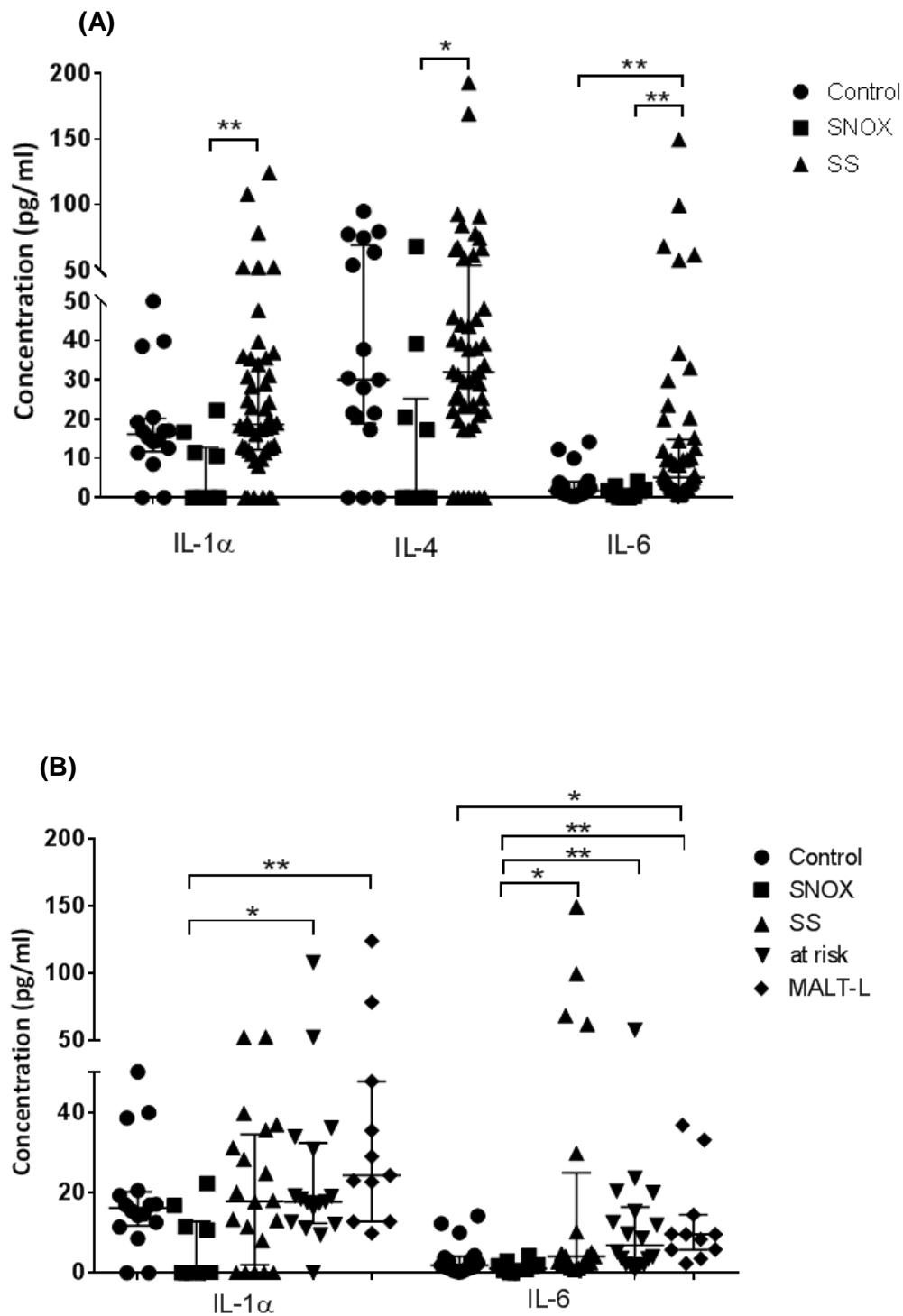


Figure 5-6 Concentration of cytokines in parotid saliva from different groups when SS groups are combined (A) and subgrouped (B) measured via screening assays. (n=76) CT; healthy controls, SNOX; sialadenitis, nodular osteoarthritis and xerostomia, SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma. Data are represented as median \pm interquartile range (IQR) and expressed as pg/ml. *P <0.05, **P<0.01(Kruskal-Wallis test followed by Dunn post hoc test).

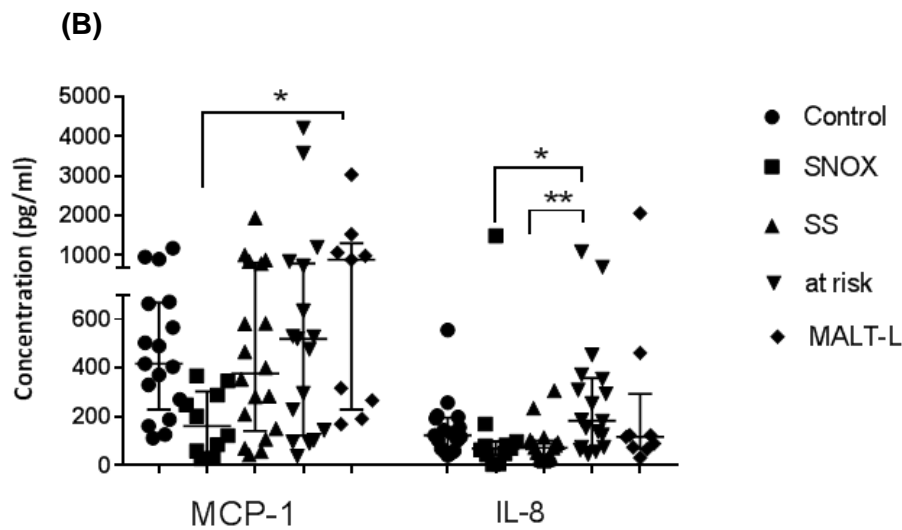
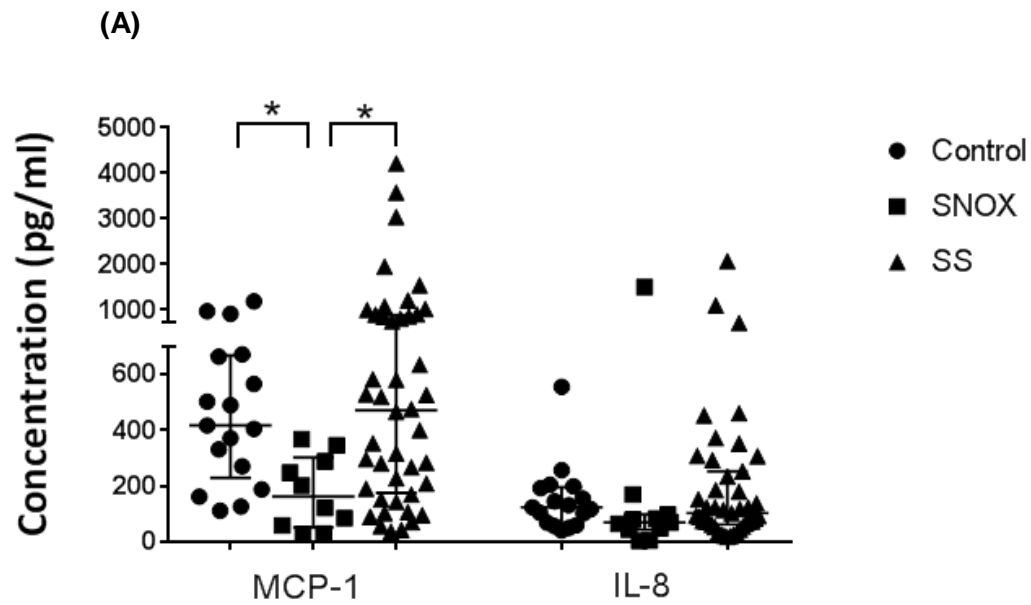


Figure 5-7 Concentration of cytokines in parotid saliva from different groups when SS groups are combined (A) and subgrouped (B) measured via screening assays. (n=76) CT; healthy controls, SNOX; sialadenitis, nodular osteoarthritis and xerostomia, SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma. Data are represented as median± interquartile range (IQR) and expressed as pg/ml. *P <0.05, **P<0.01(Kruskal-Wallis test followed by Dunn post hoc test).

Table 5-5 Salivary levels (parotid) of cytokines measured in healthy and disease subjects via Luminex screening immunobead assays

	Cytokines	Healthy controls (CT) (n=17)	Disease controls (SNOX) (n=10)	SS (n=20)	SS at risk (n=18)	SS and MALT-L (n=11)	Overall SS (n=49)
(A)		Median (Q1-Q3)	Median (Q1-Q3)	Median (Q1-Q3)	Median (Q1-Q3)	Median (Q1-Q3)	Median (Q1-Q3)
	1 IL-1 α	16.9 (12.02- 29.5)	0 (0-12.8)	17.8 (2- 34.5)	17.7 (12.3- 32.3)	24.3 (12.7- 47.7)	18.7(12.2 -35)**
	2 IL-4	30.1 (18.9- 69.11)	0 (0- 25.2)	31.63 (4.318- 45.82)	29.1 (21.8- 48.07)	39.2 (29- 74.1)	32.02 (21.52- 53.7) *
	3 IL-6	1.9 (1- 4.1)	1.1 (0.1- 2.3)	4.1 (1.9- 24.9)	6.9 (2.2- 16.4)	9.6 (5.7- 14.4)	5.2 (2.3- 14.8) **
	4 IL-8	124.1 (64.64- 195.8)	69.9 (43.93- 98.08)	72.78 (26.7- 101.4)	183.3 (74.46- 357.9) **	117.4 (70.6- 293.2)	103.9(62.82- 253.7)
(B)	5 MCP-1	417.2 (229.4- 667.5) *	161.9 (51.94- 302.9)	376.8 (140.6- 805.9)	519.6 (123.3- 787.9)	890.2 (229.1- 1301) *	470.9(174.8- 874.5)
	6 IL-1 β	0	0	0 (0- 19.8)	0 (0- 18.9)	0 (0- 19.1)	0 (0- 18.4)
	7 IFN- γ	0 (0- 3.8)	0	0	0	0	0
	8 TNF- α	0 (0- 16.7)	0	0 (0- 17.8)	0 (0- 15.3)	14.2 (0- 19.4)	0- (0- 17.8)
	9 IL-2	77.6 (40.9- 96.5)	58.5 (38.2- 89.9)	56.1 (47.6- 107.3)	84.51 (47-124.6)	82.13 (42.8- 123.1)	72.4 (46.1- 121.6)
	10 IL-3	0	0	0	ND	0	0
	11 IL-22	0 (0- 20.3)	0 (0- 5.3)	0 (0- 21.9)	0 (0- 4.6)	0	0
	12 IL-23	0 (0- 482)	0 (0- 265.3)	0 (0- 491.2)	263.7 (0- 357.3)	332.2 (0- 561.5)	252.9 (0- 441.3)
	13 IL-33	0	0	0	ND	0	0
	14 MCP-3	0 (0- 68.3)	0	0 (0- 64.2)	0 (0- 65.6)	0 (0- 11)	0 (0- 110.3)
(C)	15 ST2	0	0	0	0	0	0
	16 IL-5	ND	ND	ND	ND	ND	ND
	17 IL-10	ND	ND	ND	ND	ND	ND
	18 IL-12p70	ND	ND	ND+	ND+	ND	ND
	19 IL-17A	ND	ND	ND	ND	ND	ND
	29 IP-10	^	^	^	^	^	^

CT; healthy controls, SNOX; sialadenitis, nodular osteoarthritis and xerostomia, SS; Sjogren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma, ND; not detected, ND+; one sample detected, ^; needed further dilution. Cytokines were either showing differences between the groups (A), not showing differences (B) or not detected in samples (C). Q1; first quartile, Q3; third quartile.

*P <0.05, **P<0.01(Kruskal-Wallis test followed by Dunn post hoc test).

5.4.3 Luminex Performance Immunobead Assay

5.4.3.1 Study group

This study included 82 subjects, where parotid samples in addition to whole mouth saliva samples (n=57) were analysed. Demographics and clinical characteristics are found in **table 4-2** in chapter 4. The numbers of samples for each group differ slightly owing to low sample volume or poor performance of assay. Analytes that showed significant differences between the groups according to the screening assay were selected for this assay; (IL-1 α , -4, -6, -8 and MCP1).

5.4.3.2 Cytokine levels in parotid saliva

The median (pg/ml) levels (Q1- Q3) of IL-1 α of the groups were as follows; the overall SS group was 5.3 (2.1- 17.38) while the disease and healthy controls were 2.5 (1.8- 6.8) and 6.4 (1.6- 14) respectively. The subgroups means were 5.3 (2.31- 15.05), 6 (1.73- 33.42) and 5.1 (1.7- 60) for SS, SS at risk and SS who developed MALT-L separately. Despite the difference in their means, there was no significant difference between the groups as shown in (**figure 5-8**).

On the other hand, the mean level of IL-1 β was significantly higher (p=0.041) in the overall SS group (1.5 (0.045- 16.69)) when compared to the healthy control group (0.1 (0- 1.2)) but not the disease controls (0.3 (0- 2.6)). Subgrouping SS patients did not show any significant differences between them (1.04 (0.19- 10.48), 3.4(0.12- 18.35) and 5.6 (0- 17.84)) for SS, SS at risk and those who developed MALT-L (**figure 5-9**).

IL-4 levels of the overall SS group were significantly higher (p=0.027) than the disease control group (17.7 (10.7- 25.8) vs. 12 (6.1-15), while they were not significantly higher than the healthy controls (13 (9.5- 16.3). No statistically significant differences were noted across the groups (p=0.053), when SS was subgrouped (17.7 (9.7-26.8), 18.7 (11.6- 23.4) and 14.5 (11.9-34.2) for SS, SS at risk and SS with MALT-L (**figure 5-10**).

The levels of IL-6 showed a statistically significant difference between the overall SS group (2.5 (1.43-13.19) in relation to both healthy (1.6 (0.58- 1.95)) and disease controls (1.4 (1.3- 1.9)) (p=0.12 and 0.022 respectively). When SS was subgrouped the means for SS, SS at risk and those who developed MALT-L were 2.54 (1.42- 11.39), 4.92 (1.38- 20.15) and 2.5 (1.37- 16.54), yet again no differences were detected among the groups (pairwise) despite the overall significant difference across the 5 groups (p values were corrected via post hoc test shifting them from being significant) (**figure 5-11**) and **Appendix 18**.

Mean IL-8 and MCP-1 levels of overall SS groups (109.9 (62.51- 373.8) and 355.2 (138.7-1025)) were not significantly different than healthy controls (58.02 (35.83- 151.5) and 286 (128.7- 615.1)) or the disease controls (58.11 (43.46- 150.9) and 157.4 (106.8- 296.1)) (p=0.06 and p=0.08 respectively). Neither were the subgroups different where the means for SS, SS at risk and SS with MALT-L were 101.4 (49.76- 206.6), 196.9 (75.35- 387.9) and 93.26 (28.92- 615.6) for IL-8 (p=0.12 across the groups) and 332.6 (138- 1264), 444.4 (234.2- 936.9) and 361.4 (108.6- 1673) for MCP-1 (p=0.2) (**figures 5-12 and 5-13**).

No difference was detected between SS subject groups according to the presence or absence of another autoimmune (marked in blue and red) nor SS patients at high or low risk of developing lymphoma (marked in orange and green) as shown in all figures.

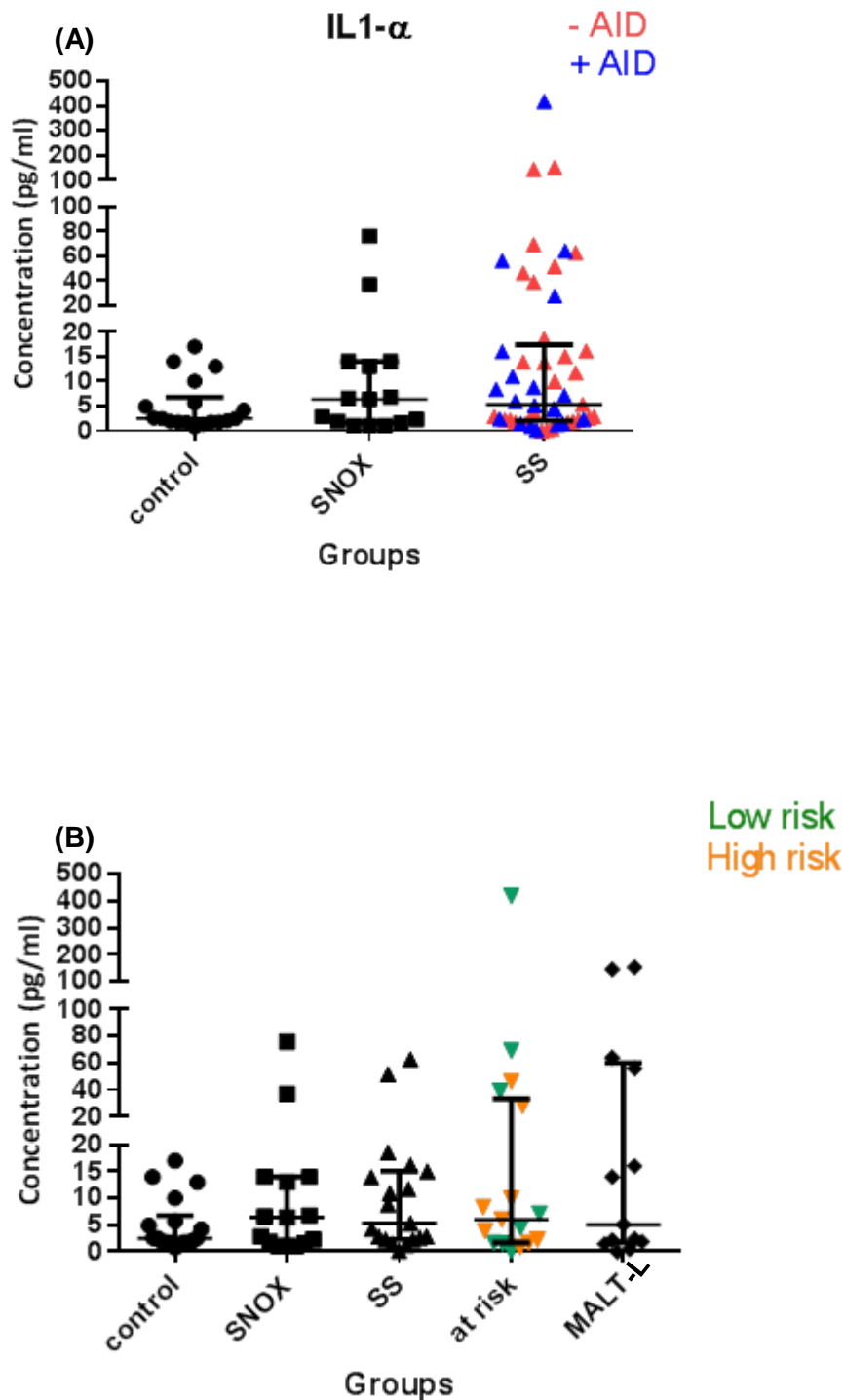


Figure 5-8 Concentration of IL-1 α in parotid saliva from different groups when SS groups are combined (A) and when subgrouped (B) measured via performance assays. (n=82) Healthy controls (n=18), SNOX; sialadenitis, nodular osteoarthritis and xerostomia (n=15), SS; Sjögren's syndrome (n=19), at risk of (n=17) or with developed MALT-L (mucosa associated lymphoid tissue lymphoma) (n=13), -/+ with or without an accompanying AID (autoimmune disease). Data are reported as median \pm (IQR) and expressed as pg/ml. (Kruskal-Wallis test followed by Dunn's post hoc test).

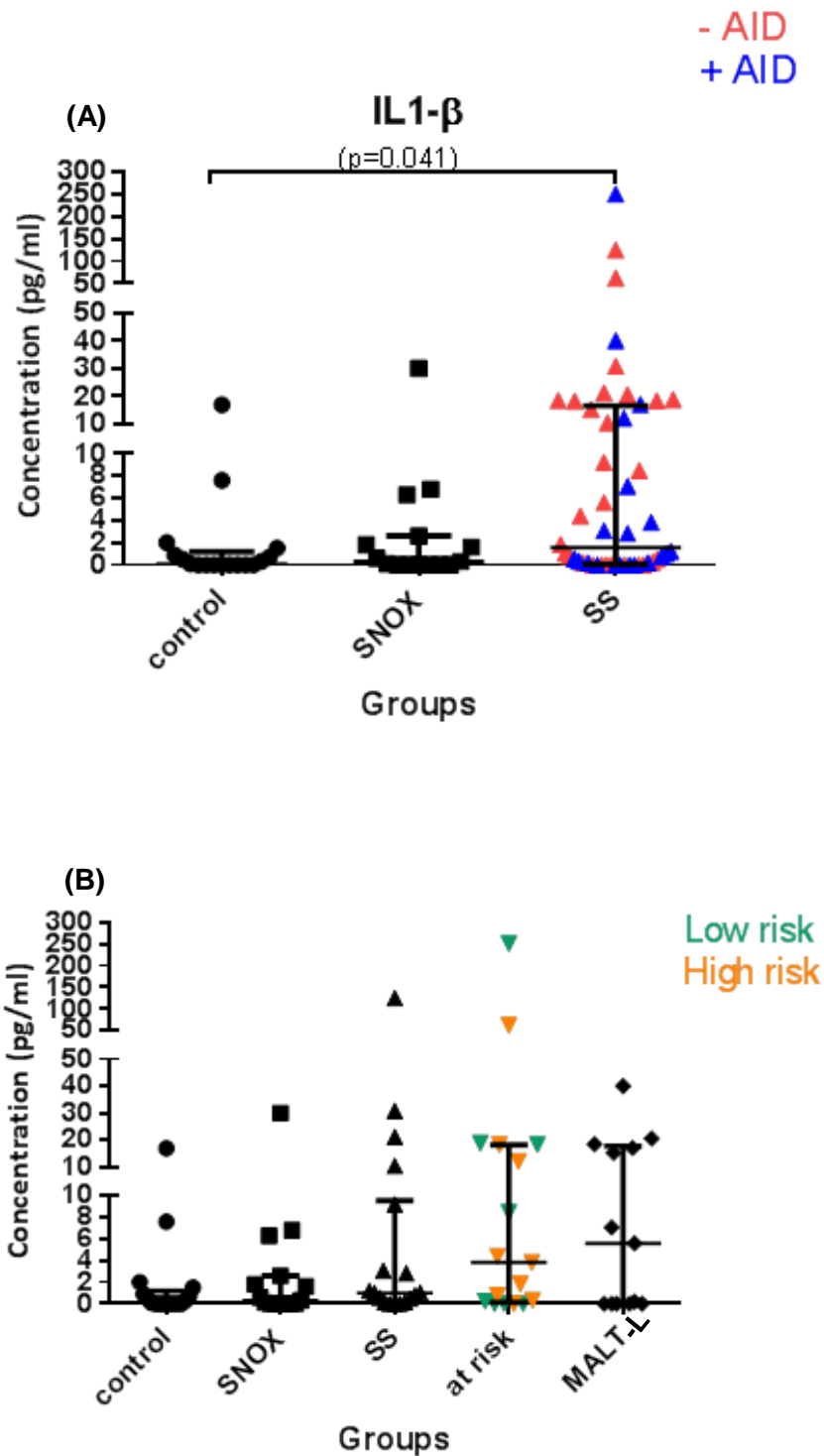


Figure 5-9 Concentration of IL-1 β in parotid saliva from different groups when SS groups are combined (A) and when subgrouped (B) measured via performance assays. (n=82) Healthy controls (n=18), SNOX; sialadenitis, nodular osteoarthritis and xerostomia (n=15), SS; Sjögren's syndrome (n=19), at risk of (n=17) or with developed MALT-L (mucosa associated lymphoid tissue lymphoma) (n=13), +/- with or without an accompanying AID (autoimmune disease). Data are reported as median \pm (IQR) and expressed as pg/ml. (Kruskal-Wallis test followed by Dunn's post hoc test).

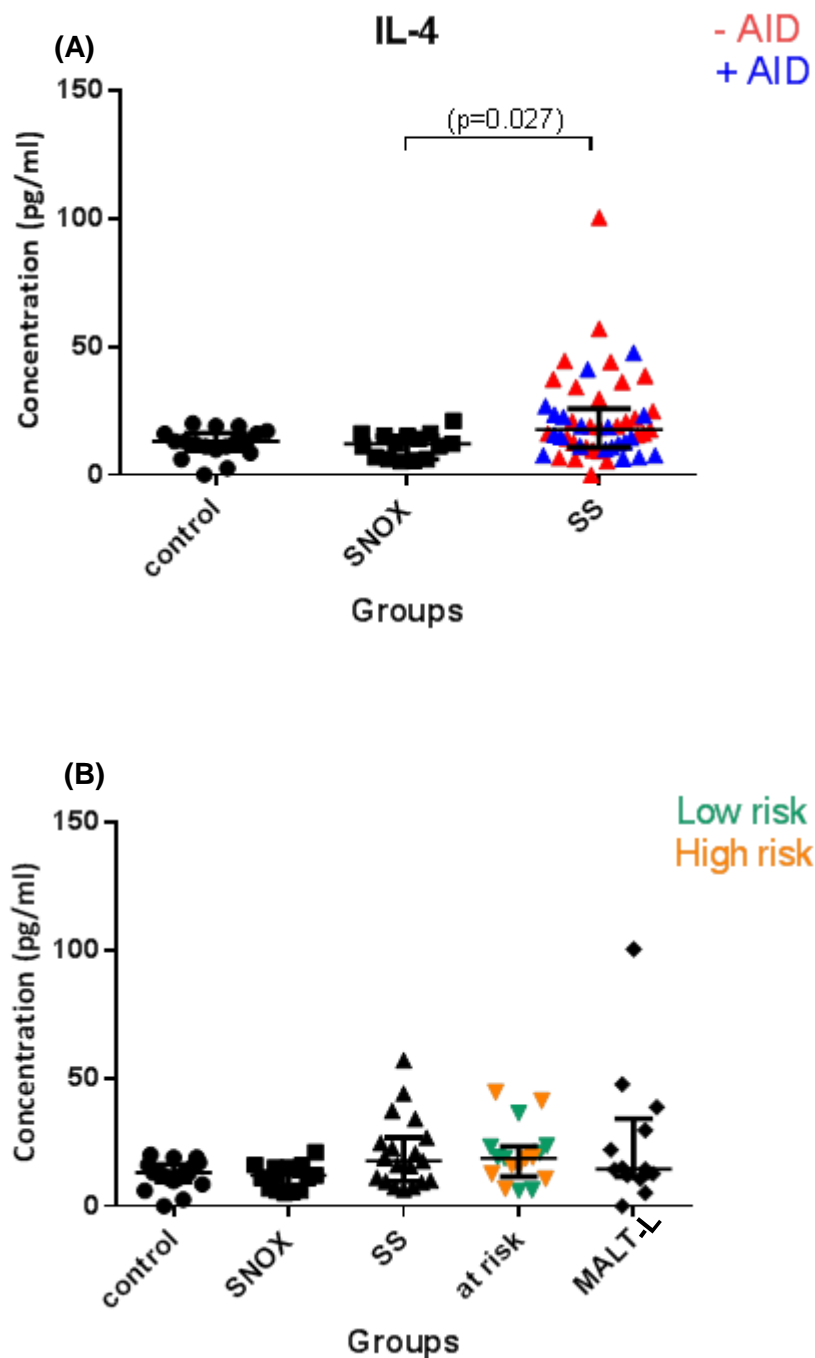


Figure 5-10 Concentration of IL-4 in parotid saliva from different groups when SS groups are combined (A) and when subgrouped (B) measured via performance assays. (n=82) Healthy controls (n=18), SNOX; sialadenitis, nodular osteoarthritis and xerostomia (n=15), SS; Sjögren's syndrome (n=19), at risk of (n=17) or with developed MALT-L (mucosa associated lymphoid tissue lymphoma) (n=13), -/+ with or without an accompanying AID (autoimmune disease). Data are reported as median \pm (IQR) and expressed as pg/ml. (Kruskal-Wallis test followed by Dunn's post hoc test).

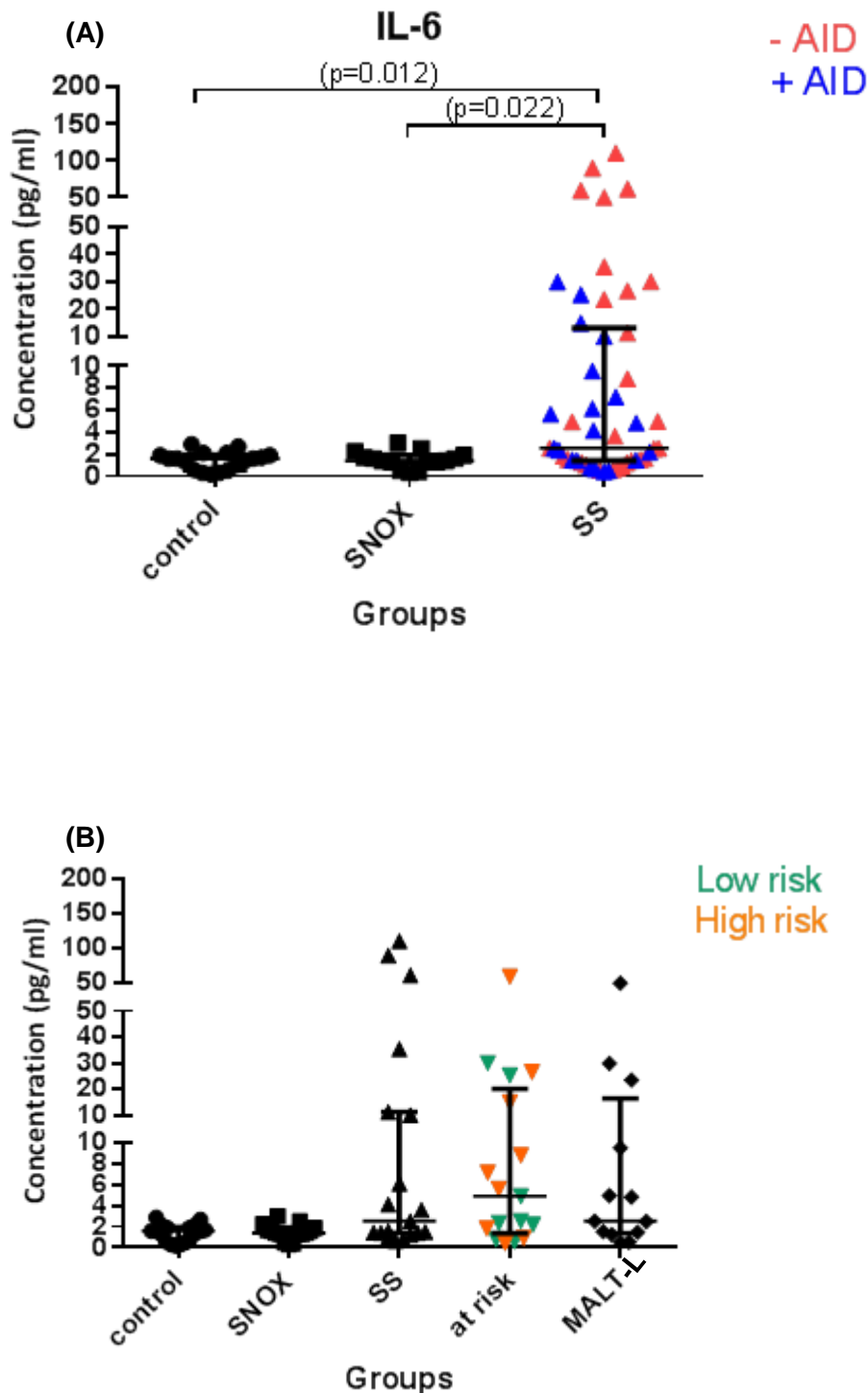


Figure 5-11 Concentration of IL-6 in parotid saliva from different groups when SS groups are combined (A) and when subgrouped (B) measured via performance assays. (n=82) Healthy controls (n=18), SNOX; sialadenitis, nodular osteoarthritis and xerostomia (n=15), SS; Sjögren's syndrome (n=19), at risk of (n=17) or with developed MALT-L (mucosa associated lymphoid tissue lymphoma) (n=13), +/- with or without an accompanying AID (autoimmune disease). Data are reported as median \pm (IQR) and expressed as pg/ml. (Kruskal-Wallis test followed by Dunn's post hoc test).

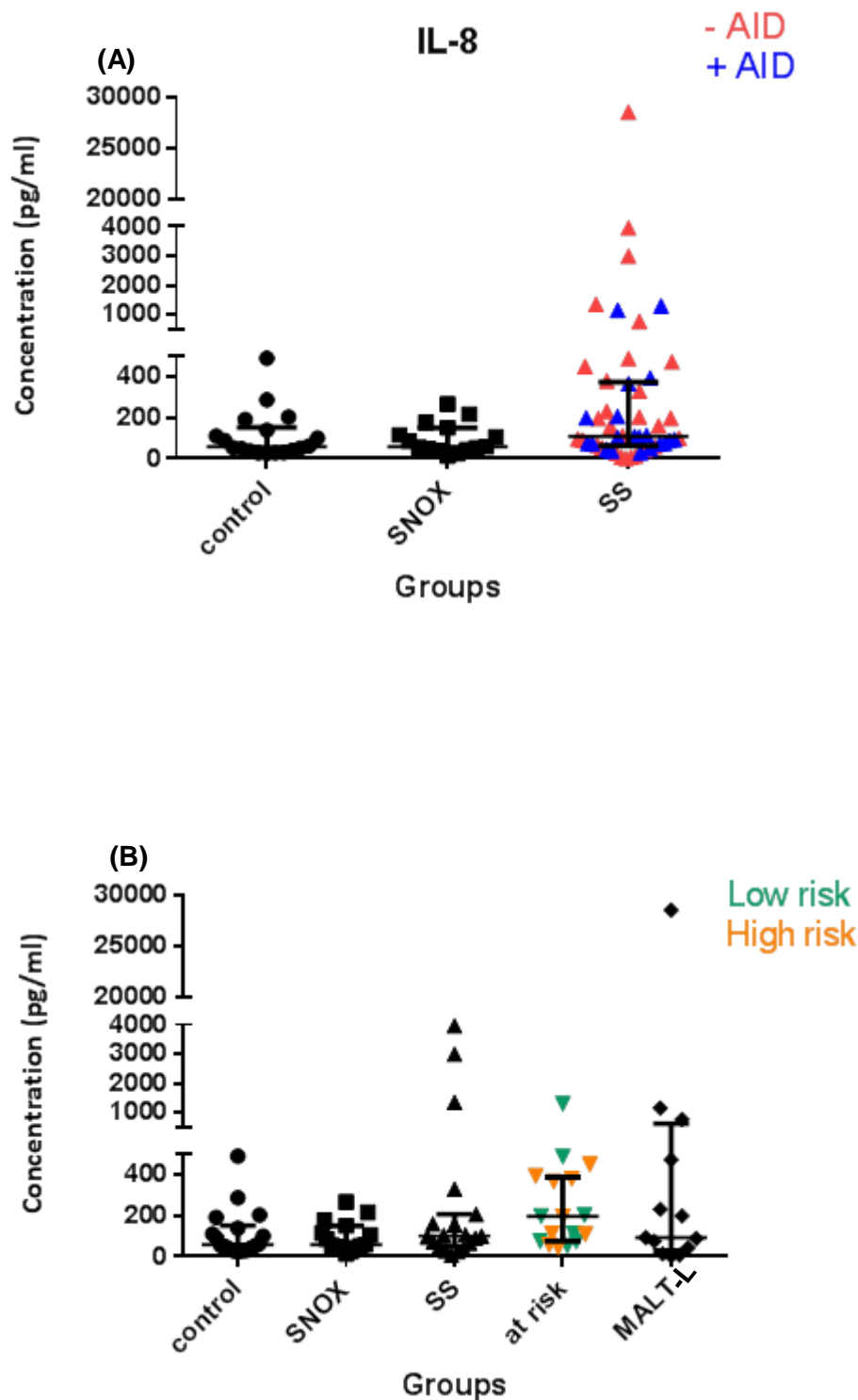


Figure 5-12 Concentration of IL-8 in parotid saliva from different groups when SS groups are combined (A) and when subgrouped (B) measured via performance assays. (n=82) Healthy controls (n=18), SNOX; sialadenitis, nodular osteoarthritis and xerostomia (n=15), SS; Sjögren's syndrome (n=19), at risk of (n=17) or with developed MALT-L (mucosa associated lymphoid tissue lymphoma) (n=13), -/+ with or without an accompanying AID (autoimmune disease). Data are reported as median \pm (IQR) and expressed as pg/ml. (Kruskal-Wallis test followed by Dunn's post hoc test).

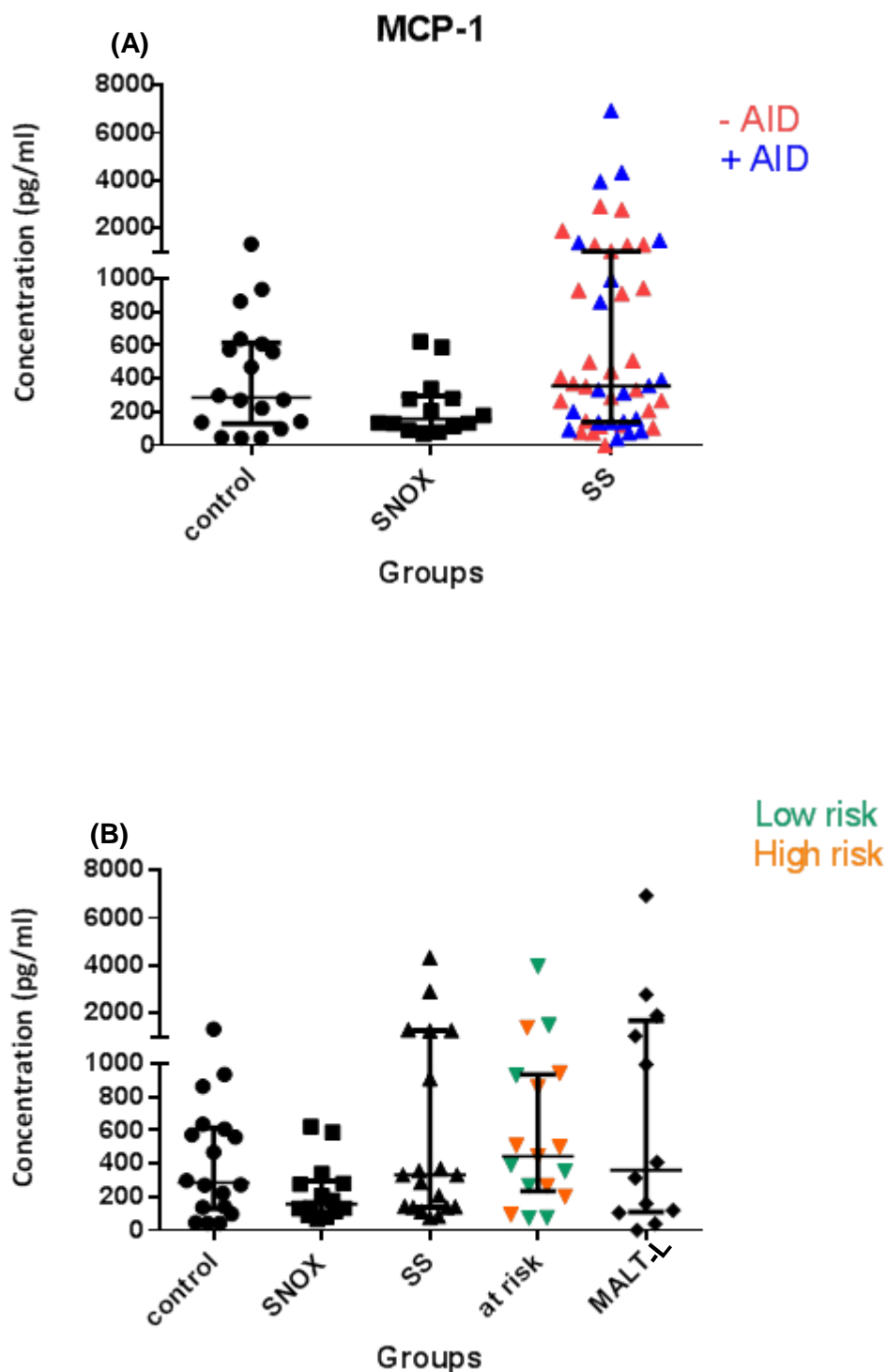


Figure 5-13 Concentration of MCP-1 in parotid saliva from different groups when SS groups are combined (A) and when subgrouped (B) measured via performance assays. (n=82) Healthy controls (n=18), SNOX; sialadenitis, nodular osteoarthritis and xerostomia (n=15), SS; Sjögren's syndrome (n=19), at risk of (n=17) or with developed MALT-L (mucosa associated lymphoid tissue lymphoma) (n=13), +/- with or without an accompanying AID (autoimmune disease). Data are reported as median \pm (IQR) and expressed as pg/ml. (Kruskal-Wallis test followed by Dunn's post hoc test).

5.4.3.3 Cytokine levels in whole mouth saliva

The concentration (median (Q1-Q3)) of IL-1 α was significantly higher in the overall SS group (951.6 (500.7- 1714)) when compared to healthy controls (476 (116- 786)) (p=0.02) but it was not significantly different when compared to SNOX (568 (244- 1036)). Only SS with MALT-L had significantly higher levels (1708 (951.6- 1720)) (p=0.03) while SS (774.5 (341.1- 1680)) and SS at risk (777.5 (481.7- 1861)) were not significantly different despite having high levels (**figure 5-14**).

On the other hand, no significant differences detected between the concentration of IL-1 β of the overall SS group (859.2 (324- 2169)) healthy (409 (164- 720.5)) and disease controls (516 (351- 1097)) (p=0.07 across the groups). While subgrouping revealed higher levels in SS with MALT-L group (2086 (732- 3614)) when compared to healthy controls (p=0.04) but the other subgroups of SS (394.1 (272.7- 1043)) and SS at risk (1127 (753.9- 2683)) did not show any significant differences (**figure 5-15**).

There was a significant difference in IL-4 levels between overall SS group (50.92 (36.9- 62.11)) and healthy (36.5 (29.75- 41.75)) as well as the disease control groups (33 (27- 43.5)) (p=0.009 and 0.04 respectively). There was an overall significant difference across the subgroups (p=0.02) but not between them where SS, SS at risk and who developed lymphoma were (46 (35.78- 57.39), 61.2 (36.6- 73.8) and 52.24 (37- 63.6)) (**figure 5-16**).

IL-6 concentration was statistically higher in overall SS group (31.2 (15.57- 64.01)) than the healthy controls (10 (5.75- 14.5)) (p=0.003) but slightly higher than the disease control (23 (4.5- 44)). Once more, SS group who developed MALT-L shows elevated levels (40.4 (15.81- 113.4)) compared to healthy controls while SS (31.22 (12.02- 51.18)) and SS at risk (27.8 (14.84- 63.78)) did not (**figure 5-17**).

Levels of IL-8 in whole saliva of overall SS patients (1930 (1057- 4878)) was significantly elevated (p=0.002) compared to healthy controls (880 (515.8- 1133)) and not significantly different than the disease controls (1361 (517.5- 2334)). While subgrouping has revealed

significant difference between healthy controls and SS at risk of developing lymphoma (4826 (1164- 5242)) as well as those who developed MALT-L (2366 (1911- 5091)) ($p=0.022$ and 0.012 separately). SS had slightly high levels (1226 (1021- 2295)) (**figure 5-18**).

MCP-1 concentration was statistically elevated in overall SS group (545.8 (289.6- 1036)) in comparison to both healthy and disease controls (278 (163.5- 442.5) and 270 (86- 357)) ($p=0.049$ and 0.014 correspondingly). Although significant change across the groups was detected ($p=0.037$) there was no significant differences detected between the subgroups SS (480.4 (322.4- 766.4)) SS at risk (1153 (253- 1791)) and SS who developed MALT-L (533 (297.3- 864.7)) (**figure 5-19**).

No statistically significant differences were found between patients with or without another autoimmune disease or between patients at high or low risk of developing lymphoma as marked on all the graphs.

Schematic presentation of the work flow done in this chapter is shown in **figure 5-20**.

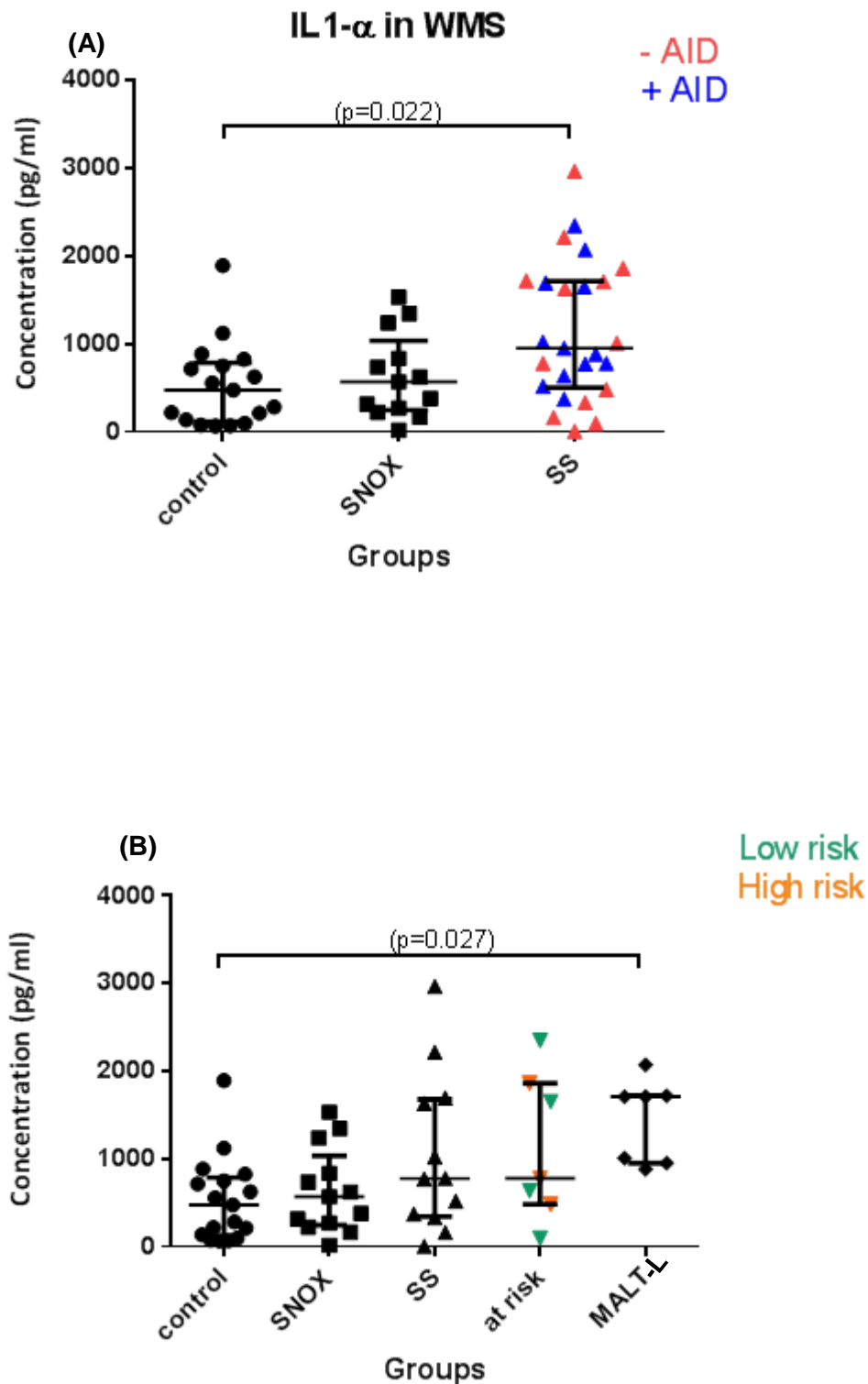


Figure 5-14 Concentration of IL-1 α in whole mouth saliva from different groups when SS groups are combined (A) and when subgrouped (B) measured via performance assays. (n=57)

WMS; whole mouth saliva, Healthy controls (n=18), SNOX; sialadenitis, nodular osteoarthritis and xerostomia (n=13), SS; Sjögren's syndrome (n=12), at risk of (n=7) or with developed MALT-L (mucosa associated lymphoid tissue lymphoma) (n=7), -/+ with or without an accompanying AID (autoimmune disease). Data are reported as median \pm (IQR) and expressed as pg/ml.

(Kruskal-Wallis test followed by Dunn's post hoc test).

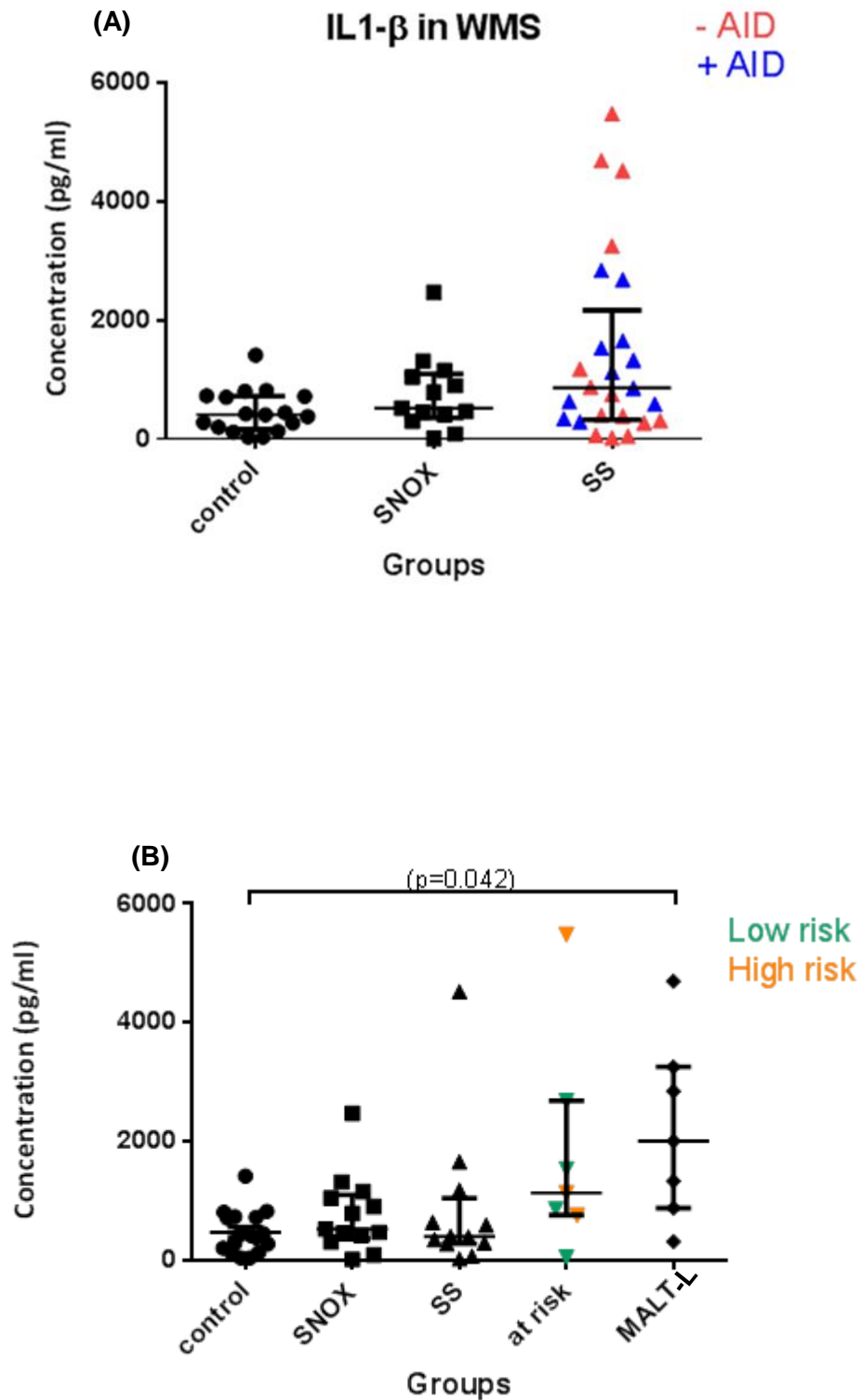


Figure 5-15 Concentration of IL-1 β in whole mouth saliva from different groups when SS groups are combined (A) and when subgrouped (B) measured via performance assays. (n=57) WMS; whole mouth saliva, Healthy controls (n=18), SNOX; sialadenitis, nodular osteoarthritis and xerostomia (n=13), SS; Sjögren's syndrome (n=12), at risk of (n=7) or with developed MALT-L (mucosa associated lymphoid tissue lymphoma) (n=7), +/- with or without an accompanying AID (autoimmune disease). Data are reported as median \pm (IQR) and expressed as pg/ml. (Kruskal-Wallis test followed by Dunn's post hoc test).

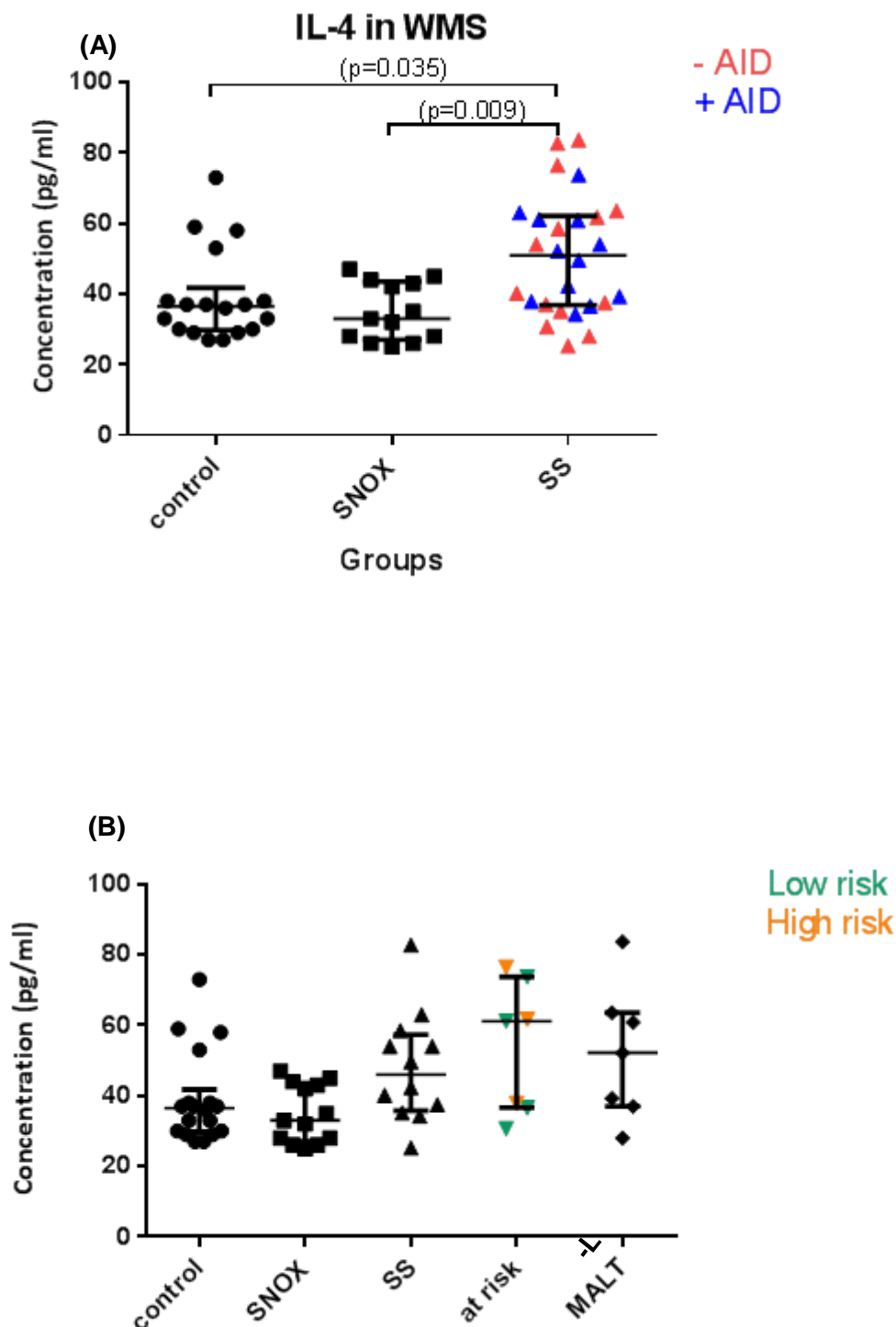


Figure 5-16 Concentration of IL-4 in whole mouth saliva from different groups when SS groups combined (A) and when subgrouped (B) measured via performance assays. (n=57)

WMS; whole mouth saliva, Healthy controls (n=18), SNOX; sialadenitis, nodular osteoarthritis and xerostomia (n=13), SS; Sjögren's syndrome (n=12), at risk of (n=7) or with developed MALT-L (mucosa associated lymphoid tissue lymphoma) (n=7), +/- with or without an accompanying AID (autoimmune disease). Data are reported as median \pm (IQR) and expressed as pg/ml.

(Kruskal-Wallis test followed by Dunn's post hoc test).

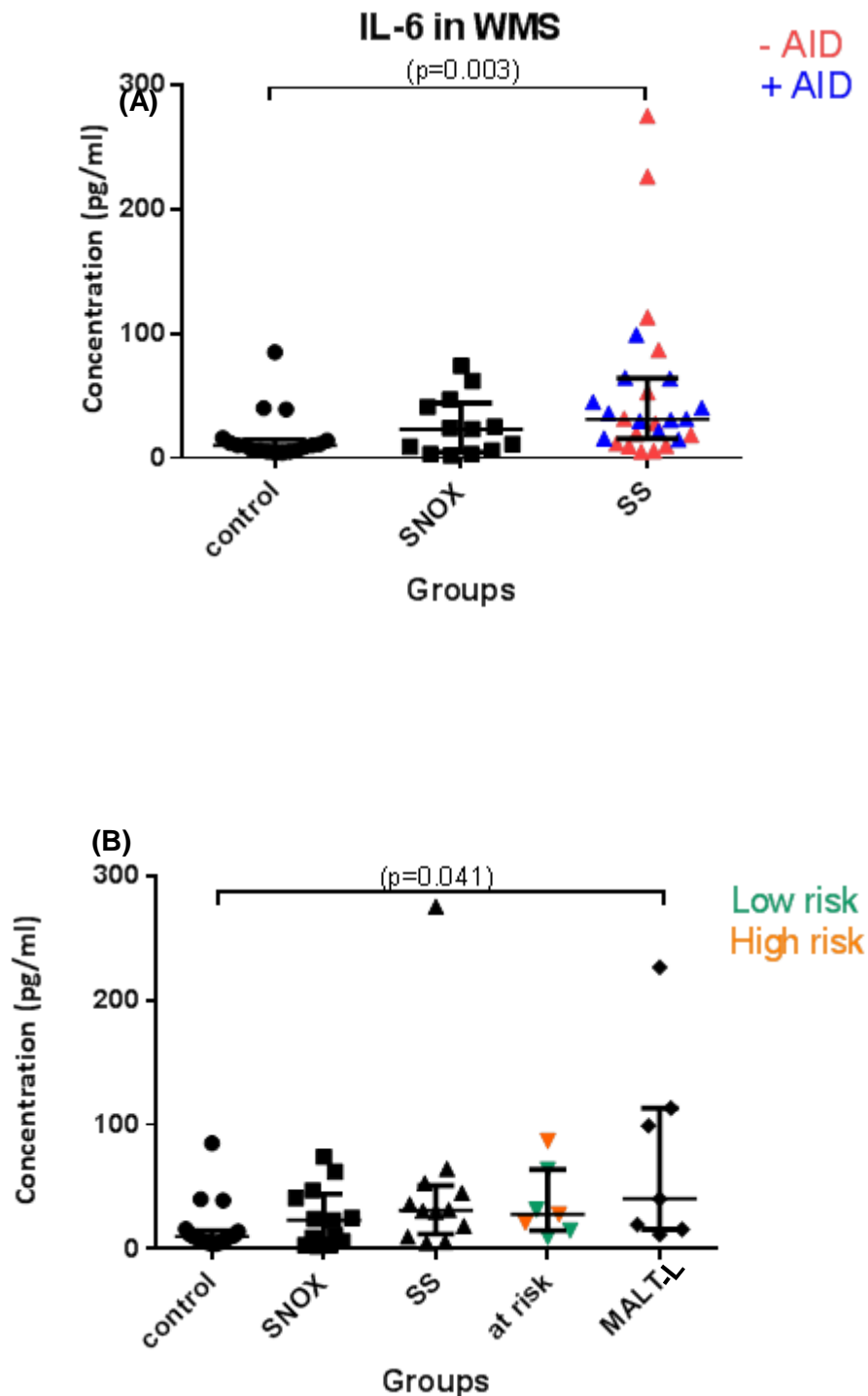


Figure 5-17 Concentration of IL-6 in whole mouth saliva from different groups when SS groups are combined (A) and when subgrouped (B) measured via performance assays. (n=57) WMS; whole mouth saliva, Healthy controls (n=18), SNOX; sialadenitis, nodular osteoarthritis and xerostomia (n=13), SS; Sjögren's syndrome (n=12), at risk of (n=7) or with developed MALT-L (mucosa associated lymphoid tissue lymphoma) (n=7), +/- with or without an accompanying AID (autoimmune disease). Data are reported as median \pm (IQR) and expressed as pg/ml. (Kruskal-Wallis test followed by Dunn's post hoc test).

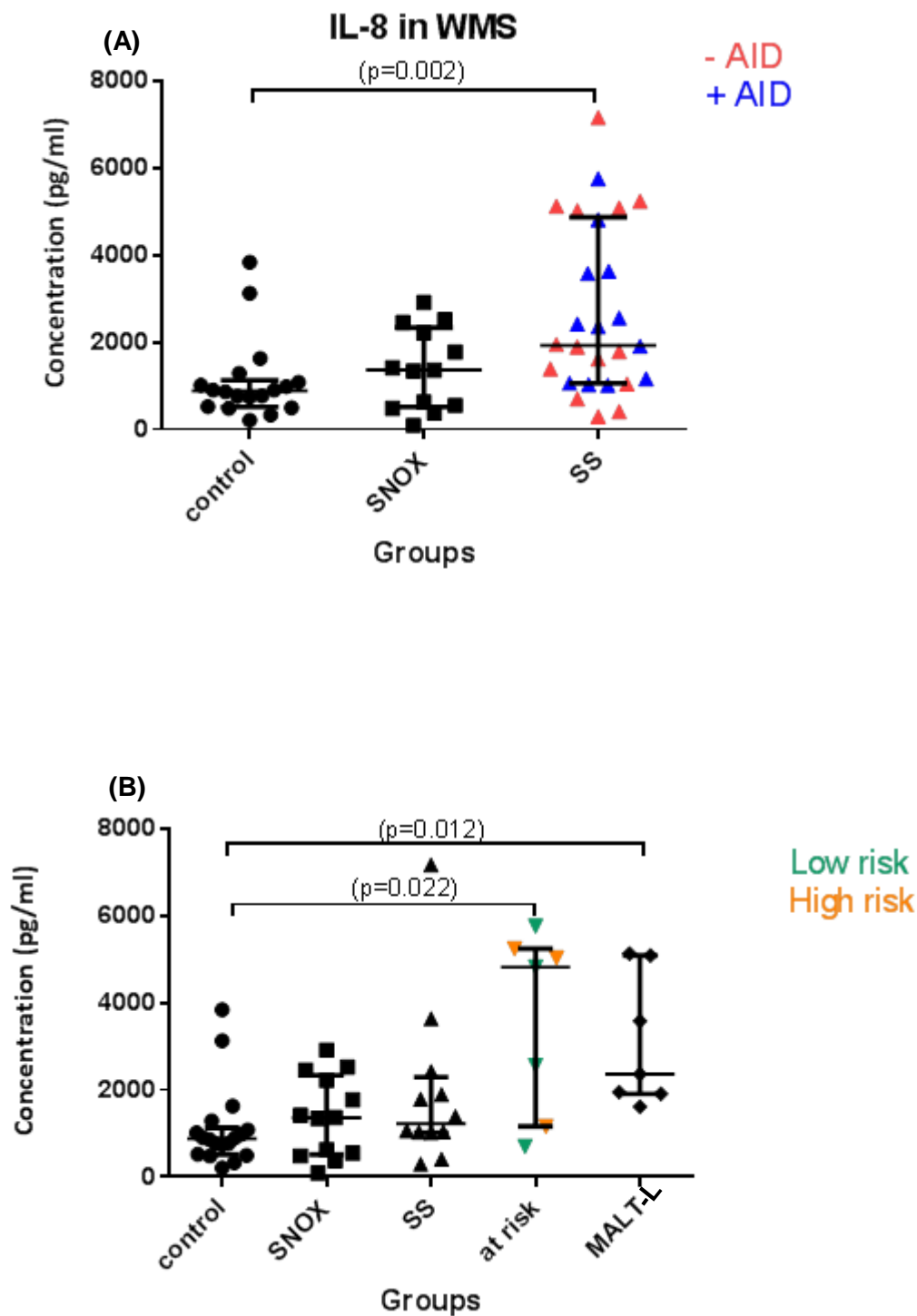


Figure 5-18 Concentration of IL-8 in whole mouth saliva from different groups when SS groups are combined (A) and when subgrouped (B) measured via performance assays. (n=57) WMS; whole mouth saliva, Healthy controls (n=18), SNOX; sialadenitis, nodular osteoarthritis and xerostomia (n=13), SS; Sjögren's syndrome (n=12), at risk of (n=7) or with developed MALT-L (mucosa associated lymphoid tissue lymphoma) (n=7), +/- with or without an accompanying AID (autoimmune disease). Data are reported as median \pm (IQR) and expressed as pg/ml. (Kruskal-Wallis test followed by Dunn's post hoc test).

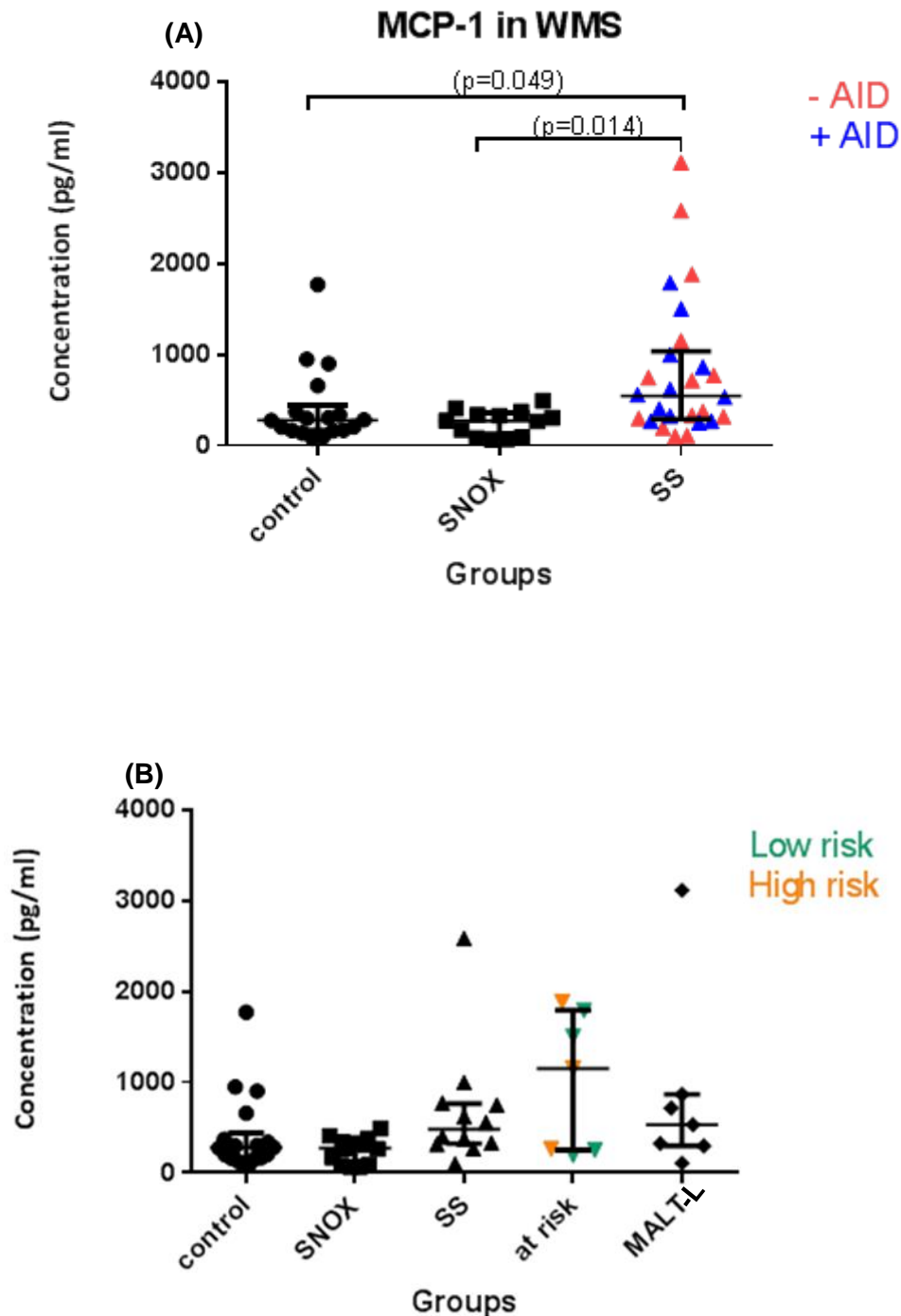


Figure 5-19 Concentration of MCP-1 in whole mouth saliva from different groups when SS groups are combined (A) and when subgrouped (B) measured via performance assays. (n=57)

Healthy controls (n=18), SNOX; sialadenitis, nodular osteoarthritis and xerostomia (n=13), SS; Sjögren's syndrome (n=12), at risk of (n=7) or with developed MALT-L (mucosa associated lymphoid tissue lymphoma) (n=7), +/- with or without an accompanying AID (autoimmune disease). Data are reported as median \pm (IQR) and expressed as pg/ml. (Kruskal-Wallis test followed by Dunn's post hoc test).

5.4.3.4 Associations of cytokines levels

5.4.3.4.1 Whole and parotid saliva levels

There was a positive correlation between parotid and whole mouth salivary levels of IL-1 α , -4, -6 and MCP-1 of the subjects group (n=57), while there was no correlation between the salivary levels of the other cytokines (IL-1 β and IL-8). A correlation was noted as well when SS subgroups were only included (n=26) for just IL- 4, -6 and MCP-1. On the other hand, no association was found in control group (n=18) except for IL-1 α , results are summarised in **table 5-6**.

Table 5-6 Summary of correlations between parotid and whole mouth salivary levels of cytokines measured by performance assays

Spearman's rho						
cytokines	Correlation Coefficient (r)	P value	Correlation Coefficient (r)	P value	Correlation Coefficient (r)	P value
Whole parotid & levels	All subjects (n=57)		SS patients (n=26)		Healthy controls (n=18)	
IL-1 α	0.273*	0.042	0.232	0.254	0.489*	0.046
IL-1 β	0.213	0.122	0.288	0.284	0.402	0.122
IL-4	0.279*	0.035	0.411*	0.037	0.044	0.861
IL-6	0.484**	0.000	0.502**	0.009	0.43	0.075
IL-8	0.157	0.244	0.12	0.559	0.284	0.254
MCP-1	0.213*	0.012	0.458*	0.018	0.022	0.93

5.4.3.4.2 Associations in relation to clinical parameters

Cytokine levels were associated with the flow rates and ultrasound scores (USS). A negative correlation was found between all of the cytokines concentrations and flow rates (parotid and whole saliva) and it was statistically significant between IL-1 α , -4, -8 and MCP-1 concentrations in parotid saliva and the parotid flow rate as well as between IL-4, -6 and MCP-1 levels in whole mouth saliva and whole flow rate. However, no correlation was found between cytokine levels in whole mouth saliva and USS but there was a positive correlation between parotid levels of only IL-6 and -8 and USS. A summary of findings is shown in **table 5-7**.

Table 5-7 Clinical parameters in relation to cytokine levels in parotid and whole mouth saliva

Spearman's rho	PFR		USS	
Parotid levels	Correlation Coefficient	P value	Correlation Coefficient	P value
IL-1α	r= -0.32**,	p=0.003	r=0.09,	p=0.57
IL-1β	r= -0.19,	p=0.1	r=0.25,	p=0.1
IL-4	r= -0.24*,	p=0.034	r=0.07,	p=0.36
IL-6	r= -0.132,	p=0.24	r=0.3*,	p=0.049
IL-8	r= -0.22*,	p=0.046	r=0.35*,	p=0.02
MCP-1	r= -0.26*,	p=0.01	r=0.11,	p=0.48
Whole mouth levels	WFR		USS	
IL-1α	r= -0.25,	p=0.07	r=0.14,	p=0.52
IL-1β	r= -0.18,	p=0.2	r=0.32,	p=0.14
IL-4	r= -0.45**,	p=0.000	r=0.1,	p=0.35
IL-6	r= -0.38**,	p=0.004	r=0.2,	p=0.34
IL-8	r= -0.23,	p=0.09	r=0.29,	p=0.17
MCP-1	r= -0.43**,	p=0.001	r=0.12,	p=0.59

PFR; parotid flow rate, WFR; whole flow rate, USS; ultrasound score. *P <0.05, **P<0.01.

5.4.3.5 Receiver operator characteristic (ROC) curve of cytokine levels

ROC curves were constructed for the data (CT vs. overall SS), (CT vs. each subgroup individually) and (SS vs. the other 2 groups individually and grouped). Overall almost all of the cytokines (4/6) in parotid saliva can differentiate between the control group and SS groups but none were significant enough to differentiate between the SS group and the SS subjects who are at risk or developed lymphoma. The same finding was found regarding cytokine levels in whole mouth saliva where all of the cytokines were able to differentiate between CT groups and SS groups but none differentiated the SS subgroups from each other. A summary of their cut-offs and sensitivity, specificity is found in **tables 5-8 and 5-9**.

Table 5-8 ROC curve analysis on cytokines in parotid saliva

Salivary biomarkers in parotid saliva					
	Cut- off	Sensitivity (%)	Specificity (%)	AUC (%)	P value
IL-1β					
CT vs. overall SS	> 0.735	60.42	65.63	67	0.01
CT vs. SS at risk	> 0.95	58.82	68.75	68	0.04
IL-4					
CT vs. overall SS	> 14.15	65.31	66.67	70	0.002
CT vs. SS/ MALT-L	> 14.69	53.85	66.67	69	0.05
IL-6					
CT vs. overall SS	> 1.60	65.31	63.64	74	0.0003
CT vs. SS at risk	> 2.51	52.94	90.91	67	0.05
CT vs. SS/MALT-L	> 2.8	53.85	93.94	81	0.001
IL-8					
CT vs. overall SS	> 88.64	65.31	60.61	65	0.02
CT vs. SS/ MALT-L	> 88.6	69.23	60.61	72	0.02

CT; healthy controls, SS; Sjögren's syndrome, SS/MALT-L; SS with mucosa associated lymphoid tissue lymphoma, SS at risk; at risk of MALT-L, AUC; area under the curve.

Table 5-9 ROC curve analysis on cytokines in whole mouth saliva

Salivary biomarkers in whole mouth saliva					
IL-1α	Cut- off	Sensitivity (%)	Specificity (%)	AUC (%)	P value
CT vs. overall SS	> 624	73.08	60	70	0.003
CT vs. SS/ MALT-L	> 858.6	100	80	90	0.0009
IL-1β					
CT vs. overall SS	> 552.6	64	60	65	0.05
CT vs. SS at risk	> 720.5	85.71	66.67	78	0.02
CT vs. SS/ MALT-L	> 844.5	85.71	80	86	0.003
IL-4					
CT vs. overall SS	> 37.25	73.08	64.52	75	0.001
CT vs. SS at risk	> 38.65	71.43	70.97	83	0.006
CT vs. SS/ MALT-L	> 42.2	85.71	74.19	83	0.007
IL-6					
CT vs. overall SS	> 14.4	80.77	64.52	74	0.001
CT vs. SS at risk	> 18.74	71.43	67.74	77	0.02
CT vs. SS/ MALT-L	> 27.49	100	77.42	85	0.004
IL-8					
CT vs. overall SS	> 1376	69.23	70.97	75	0.001
CT vs. SS at risk	> 1995	85.71	80.65	91	0.0009
CT vs. SS/ MALT-L	> 1843	57.14	80.65	76	0.03
MCP-1					
CT vs. overall SS	> 312.8	73.08	64.52	74	0.002
CT vs. SS at risk	> 316.	71.43	64.52	77	0.02
CT vs. SS/ MALT-L	> 389.	85.71	80.65	82	0.009

DCT; disease controls, SS; Sjögren's syndrome, SS/MALT-L; SS with mucosa associated lymphoid tissue lymphoma, SS at risk; at risk of MALT-L, AUC; area under the curve.

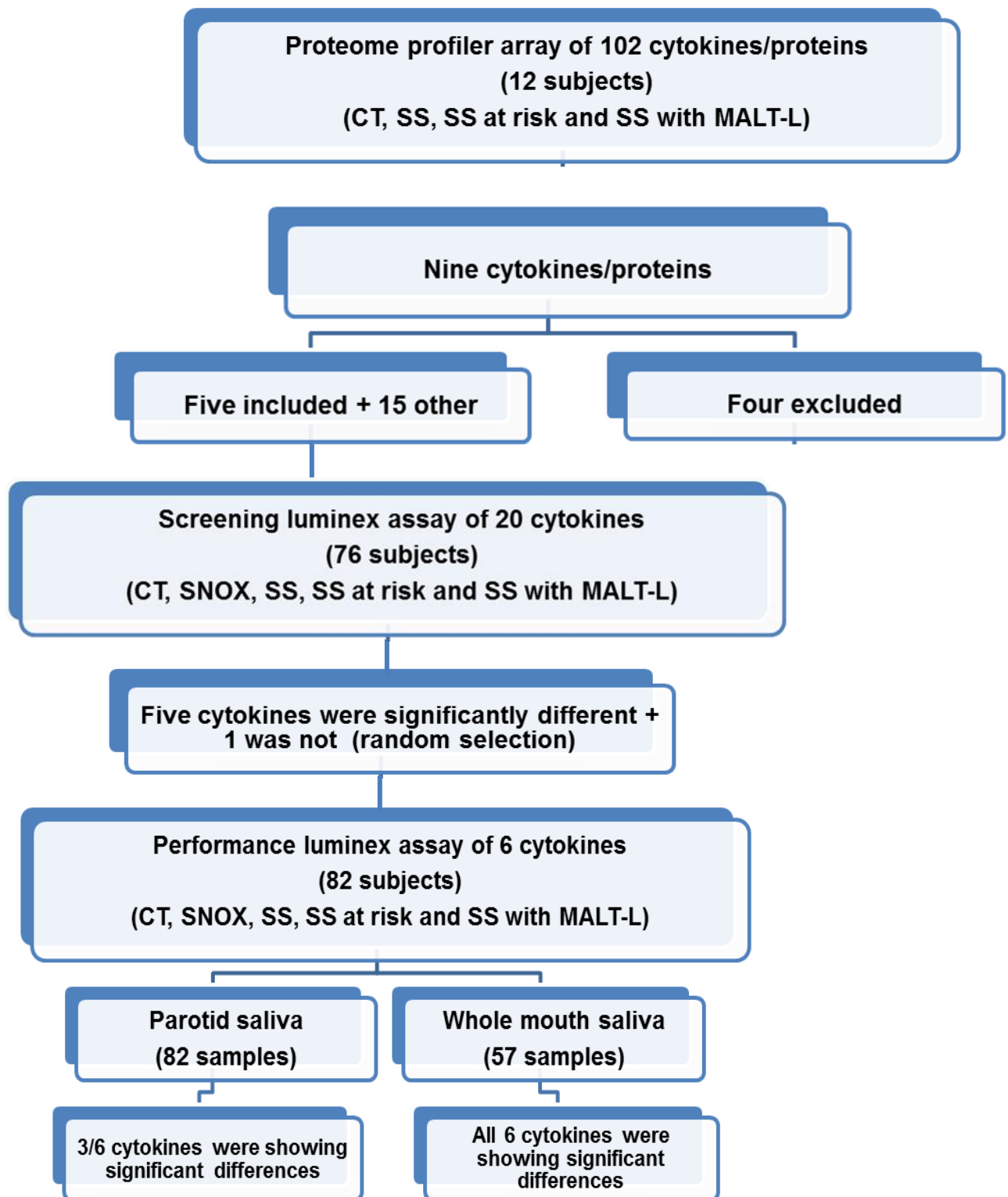


Figure 5-20 Schematic presentation of the work flow done in this study

5.5 Discussion

This study investigated whether an individual's Sjögren's syndrome (SS) phenotype could be defined on the basis of salivary cytokine biomarkers, whether it is related to risk of developing lymphoma and whether these cytokines are associated with parameters of salivary gland function. The approach taken was to screen saliva samples for a range of cytokines using different high throughput immunoassays and to try and quantify few via more sensitive assay.

5.5.1 Screening of cytokines

5.5.1.1 Cytokine antibody array

A human cytokine array was utilized to evaluate various cytokines (102) providing guidance for the subsequent analyses, to the best of our knowledge, this is the first study on the discovery of candidate cytokine markers in parotid saliva of SS patients at different subgroups using the proteome profiler approach.

It is worth noting that another study by Hu et al., (2011) have utilized the same approach; although the microarrays were designed to detect salivary autoantibodies rather than cytokines; however, the authors followed the assay by validation using ELISA similarly to the present study (multiplex assay).

The data suggested a range of cytokines were up- regulated using a cut- off of 1.5 fold change, when SS, SS at risk and those who developed lymphoma were compared to each other and to healthy controls. Cytokines were easily visualised and tracked when ranked, and in order to narrow the selected cytokines, further comparisons via ANOVA and post-hoc tests were done to increase the power of analysis (**tables 5-2 and 5-3**). Results were prioritized, and from those assigned highest priority candidate cytokines biomarkers were selected for further validation; accordingly, ST2, vitamin D BP, CD30, Endoglin, IL-10, -22, - 32 $\alpha/\beta/\gamma$, MCP-1 and -3 were the final selection (**figures 5-4 and 5-5**).

Bearing in mind the qualitative nature of this test where false positives and false negatives are easily included/excluded and the relatively limited number of samples (n=3 per group), the final

selection was viewed with caution. Reviewing the literature supported most of the findings but since the targets were primarily cytokines/chemokines; Endoglin (CD105 or TGF- β receptor), CD30 and vitamin D BP were excluded (refer to **Appendix 19** for the relevance of these cytokines in published literature). The final selection was ST2, MCP-1, -3, IL-10, -22 and -32 $\alpha/\beta/\gamma$.

The next phase was to analyse those markers via a more quantitative test that has the capacity of measuring multiple markers at the same time. Bead- based assays (screening Luminex assays) were chosen and 15 cytokines were included after reviewing the literature and a total of 21 cytokines were selected.

Unfortunately, IL-32 $\alpha/\beta/\gamma$ was not available commercially as part of the analytes supplied with the Luminex kits, thus it was excluded (leaving 20 cytokines for analysis). However, it is worth noting that this pro-inflammatory cytokine was linked to SS through its over-expression in the cellular infiltrate of minor salivary glands of SS patients in comparison to healthy controls (Sfriso et al., 2007). Also, it was correlated to degree of inflammation (Murray-Brown et al., 2013).

Interestingly Suppression of Tumorigenicity 2 (ST2) or (interleukin IL- receptor like 1) which is a Th2 related, cardiac biomarker and a specific ligand of IL-33 was found significantly increased when SS and control samples were compared to SS at risk of developing lymphoma ($p=0.007$ and 0.005 correspondingly). The ST2- IL-33 axis has a role in SS pathogenesis and it has been reported in a number of studies; Awada et al., (2014) found increased levels of both IL-33 and ST2 in SS sera when compared to controls and up-regulated IL-33 expression in salivary glands of SS patients while ST2 expression was down-regulated. Similar findings regarding the serum levels were reported by Zhao et al., (2013) where even higher levels were observed in SS patients with interstitial lung disease and correlated with Rheumatoid factor and anti-SSB levels. Another study by Jung et al., (2015) has noted similar findings to the first study where serum levels and salivary gland expression of IL-33 and ST2 were higher in SS patients reporting a reduced expression in advanced inflammatory cases and some associations to clinical parameters. A more recent study has confirmed higher serum levels and a positive correlation

with disease activity (Margiotta et al., 2016). In addition, several studies have linked IL-33 and ST2 to cancer according to a recent review (De la Fuente et al., 2015). Taken together, the studies reported indicate the necessity of exploring the levels of IL-33 and ST2 in saliva as well, thus they were selected for the next assay. While the relevance of the rest of the cytokines (IL-10 and -22) and chemokines (MCP-1 and -3) will be discussed further in detail in the following sections.

5.5.1.2 Review of literature

Exploring Sjögrens Syndrome literature has identified a wealth of research suggesting several cytokines as potential biomarkers in SS activity and progression. Cytokines were frequently measured in serum and salivary gland biopsies which might be beyond the scope of this study but worth mentioning. The selection of additional cytokines for multiplex bead assays was based mainly on their relevance in saliva from other studies whilst stating their relevance in some of the other biological samples as well. Different methods were used to measure their levels, such as ELISA and multiplexed assays. (Summary of the identified cytokines in different samples of SS patients is found in **Appendix 20 to 25**).

In regards to cytokines in saliva several studies were found, it was noticed that BAFF, TNF- α , IFN- γ , IL-1 α , -1 β , -2, -4, -5, -6, -8, -10, -12p70, -17A, -23, MCP-1, MIP-1 α , MIP-1 β , RANTES, TARC, MDC, MIG, IP-10, SDF-1 α , and BCA-1 were the most studied cytokines/chemokines. From these published studies, IFN- γ , TNF- α , IL-1 α , -1 β , -2, -3, -4, -5, -6, -8, 12p70, -17A, -23 and IP10 were selected. In addition to the analytes selected according to the cytokine antibody array results; IL-10, -22, -33, MCP-1, MCP-3 and ST2 were added. Their levels in relation to other studies will be explained fully in the bead-assay section.

5.5.1.3 Luminex Screening Immunobead assay

Several studies have used multiplex immunobead-based assays for detection of salivary cytokine biomarkers in different diseases such as oral cancer (Arellano-Garcia et al., 2008), periodontitis (Teles et al., 2009), alveolar bone loss (Ng et al., 2007), peri-implantitis (Fonseca et al., 2014), systemic lupus erythematosus (Marques et al., 2016), rheumatoid arthritis (Mirrieles et al., 2010), in even healthy individuals (Khan, 2012) and many others.

Multiplex bead-based assays were selected to measure salivary cytokines because they are fast and convenient and can measure many analytes in small volumes which is very useful for some patients with diminished saliva volume (such as MALT-L risk and MALT-L patients). Furthermore, it is based on the ELISA principle, and multiplexing itself does not affect the sensitivity of assays (dupont et al., 2005).

The results obtained from this study (screening of 20 cytokines) have revealed that several cytokines were below the lower detection limit of the assay (IL-5, -10, -17, and IL-12p70) and only one was above the higher limit of detection (IP-10), while the majority were detected at the lower end of the standard curve (**table 5-5**). Only 5 cytokines (**figures 5-6 and 5-7**) have shown differences between the groups; IL-1 α , -4, -6 and MCP-1 were increased in the overall SS group compared to the SNOX group where most of the readings were extremely low and even levels of MCP-1 in this group were lower than healthy controls. Difference in levels of IL-1 α , -6, -8 and MCP-1 were detected between SS subgroups and SNOX as well. A possible explanation for the lower levels of cytokines in the SNOX group might be the presence of mucins or substances that interfered with the detection of these cytokines via screening assays. Another more reasonable explanation is that these assays were not sensitive enough to detect them and this was confirmed by the later performance assays which extended the dynamic range of the cytokine assayed. Unfortunately, IL-10 was not detected in any sample while ST2 and IL-33 were almost not detected in most of the samples according to the screening assay which contradicted the results acquired from the cytokine array. Unlike the cytokine array, no

differences were detected in levels of IL-22 and MCP-3 and only MCP-1 continued to show significant differences between the groups.

Compared to the literature it was found that according to Bertorello et al., (2004), salivary levels of IL-10 in parotid saliva were increased in most of their SS patients (11/14) which contrasted our results obtained from our screening assays where IL-10 was not detected in all of the samples; this might be due to the semi-quantitate nature of our test (screening assays) versus more sensitive test (ELISA) used by their group. IFN- γ was increased in some patients (4/14), which is similar to our finding where we could detect it in some samples as well; healthy controls (4/17), SS patients (4/49) and none from the SNOX group. It would be worth re-testing IL-10 and IFN- γ by performance assays to ascertain these results.

On the other hand, Bertorello et al., (2004) study has reported the absence of IL-2 and -4 from parotid saliva which was different to our findings where we have detected IL-2 levels and they were not showing significant differences between the groups. In addition, differences in IL-4 levels were detected in parotid saliva by screening then confirmed by the following performance assay favouring our results. And equivalent levels of IL-6 in parotid saliva were noticed when comparing our results obtained from the following performance assays to a study done by Grisius et al., (1997). Fox et al., (1994) have reported elevated levels of their measured cytokines in parotid saliva of SS patients when compared to healthy controls. Some of them supported our findings while some did not (e.g. reporting the absence of IL-4 and -5, while it agreed with our findings for IL-5 being below the detection limit but it contrasted IL-4. In addition, IP-10 levels were found to be higher in the SS group in comparison to healthy controls (Hernandez-Molina et al., 2011).

5.5.2 Quantification of cytokine levels in SS

5.5.2.1 Parotid saliva

To assess the accuracy of the results obtained from the Luminex screening assays more sensitive Luminex performance assays were performed on a narrower selection of cytokines (IL-1 α , -4, -6, -8 and MCP1) which were chosen based on the significant differences detected between the groups according to the screening assays IL-1 β was selected to evaluate the precision of the results (**figures 5-8 to 5-13**). Data acquired from the performance assays has confirmed the differences seen in parotid saliva levels of IL-4 and -6 between the overall SS group and SNOX and only IL-6 levels of overall SS group were elevated in comparison to healthy controls, which is similar to screening assay results. Though that was not the same for IL-1 α , -8 and MCP-1 where insignificant higher levels were found. Surprisingly IL-1 β levels have shown significant differences between overall SS and healthy controls even though it did not before via screening assays which might question the readings of other cytokines and might indicate the importance of re-analysing all the cytokines via performance assays specially (ST2, IL-10, -22, -33 and MCP-3) which were significant according to the cytokine array but not via Luminex screening assay. None of the subgroups were showing significant differences but higher levels were found in all of the SS groups in comparison to controls.

It was noted that data was not as spread out as in screening assays with more comparable medians of healthy and disease groups being little higher in the healthy group (a possible explanation for the higher levels was mentioned in chapter 4). Correlation between screening and performance assays was not claimed by the company and was not advisable thus it was not presented (data not shown).

5.5.2.2 Whole mouth saliva

Given their detectable levels in parotid saliva, it was tempting to determine their levels in whole mouth saliva, thus it was done and the results have highlighted that the analysis of whole saliva can be more informative than the single gland-derived saliva. When the levels of those cytokines were measured in whole saliva via performance assays, higher levels of all cytokines were observed and this was explained previously in chapter 4. All analytes (IL-1 α , -4, -6, 8 and MCP-1) were significantly higher in overall SS group compared to healthy controls while only IL-4 and MCP-1 were higher compared to SNOX as well. IL-1 β was insignificantly higher in overall SS group when compared to both control groups. Subgrouping has revealed some significant differences in IL-1 α , -1 β , -6 and -8 levels between SS patients who developed lymphoma and healthy controls and only IL-8 levels were significantly increased in SS patients who are at risk of developing lymphoma when compared to healthy controls (**figures 5-14 to 5-19**). Some potential outliers in parotid saliva data were re-analysed while those of whole mouth saliva were not.

Taken together, the data obtained from the above-mentioned assays have shown no statistically significant differences between SS subgroups although elevated levels of most of the cytokines (in parotid and whole mouth saliva) was noted in the patients who are at risk or have developed lymphoma when compared to SS group. Increasing the sample size per group will be more informative in further studies. The low levels of cytokines in parotid saliva relate to the fact that this site is relatively free from bacteria and it might not be the major contributor of these cytokines. Whilst most of the cytokines have previously been measured in other biological tissue/fluids including whole mouth saliva of SS patients the present study is the first to measure them in parotid saliva of SS patients at different subgroups and compare with the SNOX disease control group.

Comparing our results to the literature has identified comparable mean levels of IL-6 in whole mouth saliva obtained from our assays to those reported by Rhodus et al., (1998) for both SS and healthy control groups which was higher in the SS group. Furthermore, they have found

significant differences between SS and control groups regarding IL-2 levels, while we did not measure it in whole mouth saliva. On the other hand, higher IL-6 readings than ours were reported by Tishler et al., (1999) for SS group but comparable to control group. Yet again it was significantly higher in the SS group. A similar finding was reported by Nguyen et al., (2008); IL-6 was 3 fold increased more in SS group having again equivalent readings to ours. Another study by Streckfus et al., (2001) have found elevated levels of salivary IL-2 and -6 in SS patients than the control and SS patients with another autoimmune disease. Comparison between our results to theirs was not applicable due to differences in units (pg/ml vs pg/mg). In addition Boras et al., (2004) have found elevated salivary IL-6 levels in SS when compared to controls while serum levels did not differ.

Median levels of MCP-1 in whole mouth saliva of SS patients were higher than healthy controls which reinforced by the results of a study by Hernandez-Molina et al., (2011), the medians of both groups were comparable to our values being slightly higher in their group as seen on **table 5-11**. No differences were reported between SS groups with or without autoimmune disease and this supported the findings of the present study. Furthermore, in contrast to the present study, Lee et al., (2010) have reported insignificant higher MCP-1 levels in SS group compared to healthy and non-SS sicca controls whilst IL-8 was significantly different supporting our results.

A Japanese group conducted two studies that explored the levels of multiple cytokines in SS (Moriyama et al., 2012). They have found that levels of IL-1 β , -4, -6 and -8 were higher in SS when compared to controls; this was similar to the present results except for IL-1 β which was insignificantly higher in the SS group. More recently, the same group found that IL-10 and -4 were higher in patients with strong focus scores and included 2 more groups (radiation induced xerostomia and xerostomia associated with neuropsychiatric disorders and or drugs) reporting higher levels of all of the measured cytokines (Ohyama et al., 2015). Again IL-4 was elevated in patients with high focus scores together with IL-5, -1 β and -12p70 per the previous study. Kang et al., (2011) have reported higher levels of all of their measured cytokines when SS was compared to sicca and control groups. Interestingly the above two studies have found higher

levels of IL-17 in SS compared to controls but their mean readings differed; ~1.3 pg/ml in Kang et al., (2011) vs. 420 pg/ml in Ohyama et al., (2015) for SS group. On the other hand Nguyen et al., (2008) have found no differences between groups demonstrating low levels of IL-17 (~2.5 pg/ml) in whole mouth saliva. It is worth noting that IL-17 levels were not detected in parotid saliva when screening assays were done in the present study and IL-17 levels were extremely low and almost undetectable in parotid and whole mouth saliva of a few samples when the performance assay (trial kit) was used (data not shown). It would be interesting to measure their levels in whole mouth saliva of a larger group and compare them with other studies.

Interestingly a previous study compared both types of saliva in healthy individuals and found similar cytokine levels in both while some such as IL-1 α were higher in whole mouth saliva being a pro-inflammatory cytokine that is produced by epithelial cells in which is in agreement with our results (Wozniak et al., 2002).

The above-mentioned studies are summarised in **tables 5-10 and 5-11**, that show a list of reports indicating the readings of cytokines; those studies included 3 studies on parotid saliva and 8 studies on whole mouth saliva together with results obtained from the present study for detailed comparisons. Levels of salivary cytokines stated in those studies have varied widely, and it would seem that much of this apparent discrepancy might be due to differences in saliva handling and processing. It has been suggested that mucins or related components in saliva may interfere with cytokine detection (Ng, et al. 2007, Wozniak, et al. 2002). This might explain the absence of readings for some uncentrifuged samples in the present study. The effect of centrifugation was viewed where it was noted that better readings with sufficient number of beads were obtained when samples were centrifuged (data not shown), this was found not only in whole mouth saliva but even in parotid saliva.

The role of the measured cytokines in SS pathogenesis was mentioned in chapter 1 where we have demonstrated the apparent activation of the different classes (Th1/Th2) exerting their effect at different stages of the disease; Th1 being dominant response (initiation phase) as reported by the previous studies while Th2 is involved in disease progression during the early stages of inflammation, provoking B cell activation and autoantibody production, which may diminish during more advanced phases, but possibly re-emerge in the event of lymphoma development. Furthermore, chemokines play important roles in the induction of local inflammation. It is worth mentioning that although the Th1/Th2 concept is useful in understanding cytokine networks, its direct application in an autoimmune disease such SS is difficult.

Table 5-10 Cytokine levels measured in parotid saliva of SS patients by other studies

Study	Assay	Mean		Subjects		IL-1 α	IL-1 β	IL-4	IL-6	IL-8	MCP-1
Fox et al 1994	ELISA	Mean \pm SEM	pg/ml	CT	(n=11)	35 \pm 18	-----	ND	14 \pm 7	-----	-----
				SS	(n=9)	255 \pm 42*	-----	ND	289 \pm 33*	-----	-----
Grisius et al. 1997	ELISA	Mean	Pg/ml	CT	(n=14)	-----	-----	-----	0.8	-----	-----
				PBC	(n=31)	-----	-----	-----	1.07	-----	-----
				SS	(n=15)	-----	-----	-----	16.21*	-----	-----
Bertorello et al. 2004	ELISA	-----	Pg/ml	CT	(n=26)	-----	-----	ND	-----	-----	-----
				SS	(n=14)	-----	-----	ND	-----	-----	-----
Study results	Luminex bead-based assay	Mean \pm SEM	Pg/ml	CT	(n=18)	5 \pm 1.2	3.3 \pm 1.8	12.1 \pm 1.3	1.4 \pm 0.2	112.2 \pm 28.2	418.9 \pm 84.4
				SNOX	(n=15)	12.4 \pm 5.2	3.4 \pm 2	11.6 \pm 1.2	1.5 \pm 0.2	96.8 \pm 19.4	231.3 \pm 47.8
				SS	(n=49)	27.5 \pm 9.4	19.6 \pm 7.2*	21.2 \pm 2.4*	13.8 \pm 3.4*	944.4 \pm 584.4	879.6 \pm 190.4

ELISA; enzyme-linked immunosorbent assay, Gp; group, CT; healthy controls, SS; Sjögren's syndrome, PBC, primary biliary cirrhosis, SNOX; sialadenitis, nodular osteoarthritis and xerostomia, ND; not detected, * statistically significantly different.

Table 5-11 Cytokine levels measured in whole mouth saliva of SS patients by other studies

Study	Assay	Mean or Median		Subjects		IL-1 α	IL-1 β	IL-4	IL-6	IL-8	MCP-1
Rhodus et al. 1998	ELISA	Mean \pm SEM	pg/ml	CT	(n=10)	----	----	----	11.6 \pm 2.8	----	----
				SS	(n=10)	----	----	----	41.4 \pm 7.1*	----	----
Tishler et al. 1999	ELISA	Mean \pm SEM	pg/ml	CT	(n=19)	----	----	----	12.6 \pm 6.8	----	----
				SS	(n=36)	----	----	----	200.5 \pm 43.6*	----	----
Nguyen et al. 2008	Luminex assay	Mean	pg/ml	CT	(n=19)	----	----	----	~ 10	----	----
				SS	(n=21)	----	----	----	~ 30*	----	----
Lee et al. 2010	Luminex bead-based assay	Median	pg/ml	CT	(n=25)	----	----	----	----	~ 250	~ 500
				sicca	(n=30)	----	----	----	----	~ 400	~ 400
				SS	(n=30)	----	----	----	----	~ 700*	~ 1500
kang et al. 2011	Luminex bead-based assay	Mean \pm SEM	pg/ml	CT	(n=25)	----	----	~ 0.1	~12 \pm 1.5	----	----
				sicca	(n=30)	----	----	~12 \pm 5*	~14 \pm 1	----	----
				SS	(n=30)	----	----	~5 \pm 2*	~26 \pm 7*	----	----
Hernandez-Molina et al. 2011	Luminex bead-based assay	Median (min-max)	pg/ml	CT	(n=32)	----	----	----	----	----	376.8(14.7-3502)
				pre-	(n=14)	----	----	----	----	----	1015(203-10000)*
				SS	(n=44)	----	----	----	----	----	738.8(3.2-13773)*
				SS+	(n=30)	----	----	----	----	----	1068(15.9-10802)*
				SAD	(n=49)	----	----	----	----	----	784(60.4-14277)*
Moriyama et al. 2012	CBA	Mean \pm SD	pg/ml	CT	(n=15)	----	~ 50 \pm 50	~ 10 \pm 0.1	~ 40 \pm 5	~ 80 \pm 50	----
				SS	(n=36)	----	~ 340 \pm 40*	~70 \pm 40*	~280 \pm 10*	~480 \pm 50*	----
Ohyama et al. 2015	CBA	Mean \pm SD	pg/ml	CT	(n=36)	----	~370 \pm 60	~10 \pm 20	~60 \pm 15	~480 \pm 40	----
				RX	(n=22)	----	~100 \pm 40	~2 \pm 2	~45 \pm 10	~260 \pm 40	----
				XND	(n=30)	----	~240 \pm 40	~1 \pm 1	~40 \pm 5	~500 \pm 100	----
				SS	(n=90)	----	~1040 \pm 80	~55 \pm 25	~185 \pm 40	~1120 \pm 20	----

Table 5-11(Continued)

Study	Assay	Mean or Median	Subjects	IL-1 α	IL-1 β	IL-4	IL-6	IL-8	MCP-1
Study results	Luminex bead-based assay	Mean \pm SEM		pg/ml					
			CT (n=18)	528.9 \pm 117.5	462.6 \pm 87.6	39 \pm 3.1	16.2 \pm 4.7	1108.9 \pm 221.1	408.4 \pm 100.1
			SNOX (n=12)	633.4 \pm 134	759.3 \pm 180.3	34.9 \pm 2.2	25.4 \pm 6.6	1307.3 \pm 263.7	250.7 \pm 39.6
			SS (n=25)	1154.6 \pm 164	1445.2 \pm 314.2	50.8 \pm 3.2	53.4 \pm 12.8	2696.7 \pm 378.7	810.9 \pm 151.9
		Median	CT (n=18)	-----				880	278
			SNOX (n=12)	-----				1353	270.3
			SS (n=25)	-----				1930	545

ELISA; enzyme-linked immunosorbent assay, Gp; group, CT; healthy controls, SS; Sjögren's syndrome, SS+; SS with other autoimmune disease, pre; preclinical SS, SAD; systemic autoimmune disease, RX, radiation induced xerostomia, XND; xerostomia associated with neuropsychiatric disorders and or drugs, * statistically significantly different. All cytokines in the present study were significantly different in SS groups.

5.5.2.3 Associations

Correlating whole and parotid levels obtained from performance assays has identified a positive correlation of IL-1 α , -4 and -6 when all groups were compared and that correlation was found with the SS groups in levels of IL-4 -6 and MCP-1 while IL-1 α was correlated in the healthy group. This suggests that parotid glands might be a major contributor of these cytokines especially in case of local inflammation as in SS patients. The lack of specific correlations of the other cytokines between whole and parotid saliva in matched samples from the same subjects suggests that contributions to saliva by the parotid gland can vary from subject to subject and amongst the cytokines themselves having different sources (**table 5-6**).

Relating cytokine levels with the clinical parameters has shown a generalised negative correlation between flow rates and cytokine levels in parotid and whole mouth saliva and it was statistically significant for IL-4 and MCP-1 in both types of saliva while parotid levels of IL-1 α and -8 were correlated with the parotid flow rate whereas IL-6 levels in whole mouth saliva was correlated with the flow rate suggesting that they might be related to salivary function.

Their association with ultrasound score has revealed an interesting positive correlation between IL-6 and -8 parotid levels and ultrasound scores of matched disease subjects while none of the whole mouth saliva cytokines were correlated (**table 5-7**). This might confirm their roles as mediator in inflammation specifically being of glandular origin. To our knowledge this is the first report relating glandular cytokine levels to degree of inflammation scored by ultrasound.

5.5.2.4 ROC analysis

To know the possibility of using cytokines for monitoring SS, Roc curve analysis was done and has demonstrated that the diagnostic accuracy of cytokine levels in both parotid and whole mouth saliva had rather a generalised poor-fair ability in differentiating control subjects and SS (overall and subgroups). However, IL-6 levels of parotid saliva had a good ability to differentiate between controls and SS patients who developed MALT-L with 93.9% specificity and a low sensitivity of 53% even a higher cut off in whole mouth saliva levels with a good ability to differentiate between the same groups (CT vs. SS/MALT-L).

IL-1 β , IL-1 α , IL-4, IL-8 and MCP-1 have shown a similar good accuracy in differentiating between the same groups, while only IL-4 and IL-8 could differentiate between controls and SS at risk of developing MALT-L being good for IL-4 and even excellent for IL-8. Cytokine levels could not differentiate between control groups and SS neither between SS subgroup and those who are at risk or developed MALT-L (**tables 5-8 and 5-9**).

Despite having performed the test for diagnostic accuracy, the readings (cut- offs) should be viewed with caution since the validity and accuracy has not been determined yet.

Cytokine array system and bead-based assays might become powerful tools to extensively describe the SS cytokine profile and to assess the role of these proteins in the pathogenesis of the disease. Reliable quantification of a cytokine is crucially dependent on its abundance which is true for sensitive performance assay where different concentrations were noted between different types of samples. On the other hand, an important downside of bead-based technologies such as Luminex is that by having predefined proteins of interest, researchers may introduce a major selection bias into their study but this was only partially true for our study where panels were customised based on selected cytokines and following reviewing the literature (Maecker et al., 2012).

A lot of research has been published in the last decade linking SS with cytokines and chemokines in human body fluids or in tissues. Although the mechanism(s) by which these disease indicators come to exist in saliva has not been explained fully, our findings indicate that saliva may represent a significant source of discriminatory biomarkers for local, systemic inflammation in SS. However, it is important to note that lack of specificity of these molecules for a given disease; being pleiotropic, exhibiting a very similar pattern in different clinical circumstances and their rapidly changing levels are the major limitations of cytokine measurement in practical clinical setting. Nonetheless these cytokines would be very useful in preliminary stratification of SS patients for clinical trials for example allowing the exploration of an immune, an inflammatory reaction or even a precancerous and cancerous involvement where it takes place such as in parotid glands in SS patients. Furthermore, it ought to enhance the design of special, individualized, cytokine targeted therapy for these patients (Bienvenu et al., 2000). It would be interesting to explore the cytokine levels of the other salivary glandular origins for comparisons in further studies. In addition, correlating the salivary and serum levels would be of great importance.

Furthermore, tests using ELISAs where each cytokine is measured individually could be undertaken in future studies. However it is worth noting that some studies have shown a good association between the two techniques (multiplex and ELISAs), at least for some cytokines (dupont et al., 2005, Dossus et al., 2009). While some have not (Liu et al., 2005). Similarly to inconsistencies existing between ELISA kits from different companies due to their differences in antibody pairs (dupont et al., 2005); a low association between these two techniques may be due to the different antibodies used as well. Even though the possibility of using ELISA assays would have identified additional associations between SS and cytokines measured via screening assays, in particular those with levels below the limit of detection of the assay, i.e., IL-5, -10, -17, and IL-12p70, it should be noted that if high-sensitivity assays (performance) were used for these cytokines they might have been detected and this warrants further investigation. Still it is not clear that current ELISA assays would have been better able to measure the very low levels of these cytokines than the multiplex assay used, a comparison between both assays would be of great benefit.

5.5.3 Problems encountered with methods

It was planned to evaluate the levels of the rest of the cytokines/chemokines found in the literature with the intention of focusing on BAFF, SDF-1 α , and BCA-1 due to their relevance in MALT-L via Luminex screening assays. However, the results obtained from the screening assays needed further investigation thus it was decided to proceed with the performance assays instead of doing more screening assays and it would be of great importance to study the rest of the cytokines in parotid saliva in future studies.

An attempt to extrapolate the standard curve for Luminex screening assays to involve cytokines with readings below the lower limit of detection according to the manufacture's recommended dynamic range appeared to have overestimated the differences between the groups thus it was omitted and the usual protocol (standard curve) was followed. Another attempt to evaluate the accuracy of the results obtained from the Luminex screening assays via spike and recovery tests was unsuccessful (data not shown). It is assumed that some precision is sacrificed in exchange for more flexibility in panel creation with a broader selection of analytes (20 cytokines). Despite that it is argued that the sample values generated between different lots of screening assays will be consistent to each other and would therefore be a useful tool for longer term studies.

Upon selecting the cytokines for the final Luminex performance assays, IP-10 required an additional panel which was not possible due to sample volume restriction thus it would be of great importance to measure it in future studies.

Our preliminary trials (data not shown) where some mucus plugs (from parotid glands) were tested have shown no results. Those samples were analysed in different condition; either diluted and centrifuged with ceramic beads or filtered, yet again no results were obtained, a possible explanation for some samples was due to their small volume thus they became over diluted or fixed on filter papers as for other samples it might be that they needed even more treatment. Therefore, it would be most interesting to find a way to treat these samples and successfully analyse them in future studies.

Chapter 6: General discussion and future work

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6 General discussion and future work

6.1 General discussion

In Sjögren's syndrome, functional assessments such as salivary flow and ultrasound are amongst the inter-related factors which describe glandular hypofunction. Because it represents an ideal milieu for diagnosing a disease that affects the salivary glands, there is a great interest towards using saliva itself as a diagnostic fluid for SS. Parotid saliva is of great importance in cases of longer disease duration and those with or at risk of MALT-L. Although some of these clinical parameters and salivary biomarkers have been investigated before, researches have focused on one clinical aspect at a time and involved whole saliva rather than individual glandular saliva, this thesis has explored these clinical parameters and biomarkers and comparisons were done between whole and parotid gland saliva. The novelty of this thesis relies on analysing different SS subgroups and comparing them to a SNOX disease control group which has not commonly been reported by other studies. MALT-L is one of the most serious manifestations of SS thus identifying/predicting it is of great significance and this was examined throughout the thesis where patients who were defined as being at risk of developing lymphoma and those who had already developed it were compared to SS patients.

The overall aim of this thesis was to enable a better understanding of the disease activity and progression via identifying different biomarkers whether they were clinical or laboratory/sialochemical biomarkers.

6.1.1 Clinical markers

Chapter 3 investigated the potential use of the salivary gland assessment tests (WFR, PFR, CODS and USS) in discriminating SS and non- SS sicca patients as well as to differentiate between the different subgroups of SS and determine whether there is an overall association between them. All parameters of the overall SS group showed a significant difference when compared to disease controls attributed mainly to the advanced subgroups of SS (SS at risk and MALT-L groups) which showed even differences when compared to the SS subgroup

mainly the MALT-L group in all parameters while at risk group was showing a difference in USS and WFR but not the others. No differences were detected between SS subgroup and disease control group regarding WFR and PFR which is in agreement with other studies (van den Berg et al., 2007, Osailan et al., 2012, Ohyama et al., 2015, Billings et al., 2016). Differences were detected between SS subgroup and disease controls regarding CORDS similarly to Osailan et al., (2012) and USS as well and the latter is a novel finding. All of the parameters showed no differences between at risk and MALT-L groups. There was a strong negative correlation observed between CORDS and flow rates and a positive correlation with USS suggesting the reliability of using CORDS as a sensitive tool that can be easily done and this was supported by another study (Osailan et al., 2012). The positive correlation between WFR and PFR was found which is a similar finding to Vissink et al., (2003) and Kalk et al., (2002a), while it contrasted other studies (Kalk et al., 2001a, Kalk et al., 2002c, van den Berg et al., 2007). These studies have found no relation between flow rates attributed to the involvement of parotid glands in the later stages but in our study a lot of the patients were considered at their later stages and the correlation was stronger in case of the advanced stages of SS. A moderate negative correlation was noted between both flow rates and USS reflecting an association between the output of the gland and its clinical picture. The diagnostic accuracy of USS and its relation to focus score of biopsy results was investigated. It can be concluded that USS would be the ideal non-invasive test to discriminate and monitor SS patients in general (showing high sensitivity and specificity) replacing sialography and scintigraphy with a good correlation and agreement with focus score in concordance to others (Cornec et al., 2013, Delli et al., 2015, Jousse-Joulin et al., 2016, Astorri et al., 2016). The present study suggested a score of 4 was found to be an optimum cut- off that could differentiate SS from non- SS patients while higher scores usually reflecting advanced stages. A longitudinal study on dry mouth patients at 5 and 10-year time-points was completed to investigate worsening in salivary function over time. In patients with a longer disease duration it SS is a slowly progressing disease and this was proven in this follow- up study where all of the parameters remained relatively the same over the period of 5 years although some

individuals may display flare ups and remissions as in other rheumatic diseases which is in agreement with other studies (Bouma et al., 2003 , Theander et al., 2005, Pijpe et al., 2007, Haldorsen et al., 2008). The small number of patients involved in the 10-year follow- up study precludes definitive conclusions but the result was in agreement with other studies showing no significant differences between time 0 and 10 years later (Gannot et al., 2000, Haga, 2002). Although these clinical markers might be able to differentiate between different SS subgroups at one point (cross-sectional), the lack of change in clinical parameters in the longitudinal study suggests that these clinical biomarkers may give limited utility in identifying patients at risk or having MALT-lymphoma.

6.1.2 Sialochemical biomarkers

Initially a proteomics analysis of saliva was performed since this approach can identify novel potential protein biomarkers which might differentiate SS patients from those who are at risk of developing MALT-L and discriminating both disease groups from healthy controls. The proteomics data generated by LC-MS/MS analysis of both runs suggested that a range of proteins were up-regulated and down-regulated when different subgroups of SS were compared to healthy controls; focusing on the logged transformed cut- offs and the up-regulated proteins in both disease groups (SS and SS at risk) when compared with healthy controls, Actin cytoplasmic 2, Ig γ -1 chain C region, S100-A8, and S100- A9 were selected having considerable potential as biomarkers as reported previously (Al-Tarawneh et al., 2011, Katsiogiannis and Wong, 2016). Further selection of candidate biomarker was based on having cut- off of ≥ 2 when SS patients were compared to SS patients at risk in both runs, thus S100-A8, and S100- A9 were the final selection and S100A8/A9 heterodimer was chosen. Candidate biomarker was verified by ELISA; stratification of different stage of SS (SS, at risk and MALT-L subgroups) as well as differentiating them from healthy and disease control (SNOX) groups. S100A8/A9 ELISA analysis of parotid saliva supported the proteomics findings showing significant differences between overall SS group and both disease and healthy controls. Although subgrouping SS has revealed that only SS patients at risk and those

who already developed MALT-L were increased when compared to healthy controls only but not disease controls while SS subgroup has shown no significant difference. S100A8/A9 ELISA analysis of whole mouth saliva has revealed a no significant difference in the overall SS group when compared to both healthy and disease control groups showing similar values to Muller et al., (1993) and Sweet et al., (2001). Similar trends were found by another group who reported values that were 10 fold higher than ours which might be due to different sample collection and handling (Cuida et al., 1993, Brun et al., 1994, Cuida et al., 1995, Cuida et al., 1997). Only SS who already developed MALT-L subgroup has shown a difference when compared to both healthy and disease control groups as well as to SS sub group suggesting its important link to cancer development (Salama et al., 2008, Bresnick et al., 2015). Investigation of the association between S100A8/A9 and overall salivary gland function revealed a weak negative correlation between PFR and S100A8/A9 parotid levels. However, no association was found between WFR and S100A8/A9 levels in whole saliva which may be due to other contributions to levels in WMS. USS showed no correlation to both saliva (parotid and whole) levels. A strong correlation was found between parotid and whole saliva levels of S100A8/A9 in SS patients suggesting a salivary gland origin or neighbouring tissue source since the glands are the major site of inflammation in this group. On the other hand, controls showed no correlation. Proteomics is less effective in identifying cytokine biomarkers which tend to be present at lower levels in saliva and other biofluids. Therefore, potential parotid salivary cytokine biomarkers were examined using a cytokine antibody array approach to identify SS sub groups (SS, SS at risk and already developed MALT-L) and to differentiate them from healthy controls. Using a cut- off of 1.5 fold change and p- value, a range of cytokines were up- regulated when SS, SS at risk and those who developed lymphoma were compared to each other and to healthy controls (9 cytokines/proteins and 5 cytokines were included). Further screening of the selected cytokines as well as other cytokines (relevant to literature) was performed and levels were compared in SS subgroups to healthy and disease controls (SNOX) via bead- based multiplex antibody assays. Most of the cytokines were at the lower end of detection showing no differences between the groups but IL-1 α , -4, -6 and

MCP-1 showed differences between the overall SS group and SNOX while only IL-6 was increased in comparison to healthy controls as well. The cytokines have shown differences with controls when SS was subgrouped as well as for IL-8. No significant difference was found between all of SS subgroups, a tendency toward increased levels of salivary cytokines was noted in MALT-L sub group. Verification of the previously significant cytokines while adding one more cytokine (IL-1 β) from the non-significant group (total 6 cytokines) using more sensitive assays (bead- based performance) on a slightly larger number of samples yet similar groups (healthy, disease controls and all three SS subgroups) was done with comparisons between parotid levels and whole mouth levels. Some cytokines such as IL-6, -4 continued to show significant difference compared to SNOX and IL-6 compared to healthy group as well. Interestingly IL-1 β showed significant increase of the overall SS group. Subgroups of SS showed no differences when compared to other control groups or between each other, however, a tendency toward increased levels was observed in MALT-L subgroup. All cytokine levels in whole mouth saliva were significantly increased in the overall SS group compared to healthy control this time while only 2 cytokines were increased in comparison to SNOX (IL-4 and MCP-1). Subgrouping of SS has revealed several differences especially in the MALT-L subgroup. Despite using different techniques a number of studies reported similar findings (Fox et al., 1994, Grisius et al., 1997, Tishler et al., 1999, Bertorello et al., 2004, Nguyen et al., 2008, Lee et al., 2010, Kang et al., 2011, Moriyama et al., 2012, Ohyama et al., 2015). The association between those 6 cytokines with overall salivary gland function as well as between its parotid and whole saliva levels was investigated and generalised negative correlation between flow rates and cytokine levels in parotid and whole mouth saliva was observed. A positive correlation between ultrasound scores and IL-6 and -8 levels in parotid saliva was noted while none of the whole mouth saliva cytokines were correlated suggesting that parotid cytokines were mediators in inflammation specifically of glandular origin. Some parotid and whole salivary cytokines levels correlated while others did not suggesting that contributions to saliva by the parotid gland can vary from subject to subject and that glands might be a major contributor of correlating cytokines especially in case of local inflammation

as in SS patients ,which might be linked with disease activity in different subjects. It is worth noting that levels of cytokines and S100-A8/A9 were detected in patients with previous lymphoma, in most cases in complete remission. This increase in saliva level could be, at least in part, not related to the activity of the lymphoma, but could be constitutional and persist after the lymphoma was cured which could be genetically linked. The diagnostic accuracy for each test mentioned in all of the chapters was done establishing a test threshold for disease detection by providing test sensitivity and specificity. Therefore, in this study we used Receiver Operating Characteristic (ROC) curve in order to determine the best cut-off point for a given biomarker values to generate an optimum cut-off that provides a maximum sensitivity and specificity. The differences between the levels of different markers measured in parotid saliva, could not be attributed to an increased protein concentration in patients with deficient saliva, because their protein concentrations were similar in all groups (**figure 4-11**, chapter 4). Although protein concentration of whole mouth saliva did differ between the groups (especially SS with MALT-L) (**figure 4-11**) a comparison of protein S100A8/A9 levels between the groups was done after adjusting the values to ng/mg and showed no major differences between those obtained via ng/ml. Cytokine values were not adjusted thus their levels for MALT-L group might be related to the high total protein concentrations of saliva and possibly reduced fluid secretion. The biomarker studies have a limitation since patients may have been under immunosuppressor therapy. This notwithstanding, the studies showed that no difference was found between treated and untreated patients but it might be due to the insufficient power of the study to determine an effect of drugs in this rather small and heterogeneous group of patients. However, the difference between treated and untreated patients was not verified regarding the clinical parameters (chapter 3). Studies that have been carried in this thesis were the first that have compared the levels of salivary cytokines and S100-A8/A9 in different SS subgroups; at risk and those who developed MALT-L in SS patients as well as in SNOX patients, that contribute to a better understanding of disease activity and progression. The applicability of sialometry and sialochemistry as diagnostic instruments varies in different

clinical SS subgroups. While the former might be informative in case of advanced disease, the latter is often useful in the early phases of the disease.

In the present studies, we have focused on the advanced subgroups of SS indicating that both sialometry and sialochemistry are useful tools; proteomic analysis, cytokine array system and screening bead-based assays are becoming powerful tools to extensively describe the SS protein/cytokine profile assessing their role in SS pathogenesis. While ELISA remains one of the gold standard tests in analysing biological samples, multiplex assays might offer several advantages as well exhibiting a good correlation with ELISA. One of the more captivating aspects in the search of salivary biomarkers is information achieved from oral saliva and immunologic factors. Therefore, we found it of importance to study the salivary glands and their secretions to better understand the inflamed processes involved. Although the mechanism by which these disease markers come to exist in saliva has not been clarified fully, our findings suggest that it may represent a significant source of discriminatory biomarkers for Sjögren's syndrome. Yet further investigations should be addressed.

6.2 Future work

This thesis has shed light on the potential use of multiple biomarkers in differentiating SS subgroups. It can be taken further by focusing on the patients at risk group in order to understand the mechanisms behind the observed compositional changes. Furthermore, an assessment of a possible relationship between individual salivary components such as the SA100A8/A9 and candida count might provide a further insight as to whether this biomarker is of significant functional relevance.

Moreover, the results of this thesis give rise to further areas that need investigation:

1. Further analysis of the variations in the parameters (WFR, PFR, CODS and USS) assessed in this thesis are necessary and the inter- and intra- individual and observer variability should be considered in order to estimate whether differences observed are the result of individual variations of the examination technique or real individual variations.
2. The diagnostic accuracy of USS should be investigated on a larger cohort of patients and further its correlation with histology especially major salivary gland biopsy assessed in order to further reinforce results obtained from the present study indicating its usefulness as a simple non-invasive test. Different USS scoring systems should be compared in order to develop a consensus with consistent procedures.
3. Further follow- up longitudinal studies of greater than 10 years on larger group of patients are required in order to establish whether there is functional loss over time.
4. Further studies of more SNOX patients compared with SS are required in order to further verify it as a distinct disease entity.
5. Considering the presence of other disease modifying factors such as the effect of treatment on the outcome measures; differences between treated and untreated patients should be studied fully. Among the most used treatments; steroids, immunosuppressants and hydroxyquinoline would be included. Patients would be categorised according to the duration of treatment. Standardization of sample

handling, collection and processing is still required for further studies and special attention to the effect of centrifugation would be of great importance.

6. Further analysis with different proteomic methods would achieve more reliable results especially for validating salivary biomarkers an example of which would be Tandem-mass-tagging or labelling methods. An integrated proteomic analysis of the glandular tissue or another exocrine secretion such as tears should be performed and compared with saliva to investigate the differences between their pathological involvements.
7. The relationship of salivary S100A8/A9 with the focus score and possibly tear fluid S100A8/A9 levels should be clarified to better understand SS glandular disease activity as well as analysing other glandular salivary S100A8/A9 levels (e.g. submandibular/sublingual glands) in order to identify the possible analyte origins and to compare the differences between the glandular secretions. Individual levels of S100-A8 and -A9 should be analysed in order to determine their relative abundance in relation to the heterodimer of both.
8. Performing cytokine array tests on whole mouth saliva with comparison to results obtained from the multiplex bead- based assays would give some insight about the reliability of these tests as first-choice screening tests. Re-testing cytokine levels (showing low levels or 0 values) which were tested via screening bead- based assays via more sensitive bead-based assays to ascertain their levels. Testing the accuracy and reproducibility of bead- based assays is required via spike and recovery tests in order to verify the applicability of using these tests for further research. Special interest in measuring BAFF, SDF-1 α , and BCA-1 levels in parotid saliva would be of great importance since they were frequently linked to advanced subgroups of SS. Re-measuring chemokine levels especially CXCL10 (above the detection limit) would provide further insight to the extent of chemokine involvement. Considering other salivary/exocrine sources of cytokines will further aid in understanding of their pathogenic roles. Re-analysing the same tests specifically ELISA and bead-based

performance tests could be performed on a larger cohort of patients in order to improve the reliability of these tests and demonstrate their utility.

9. It may well be that a multifactorial model including salivary biomarkers and clinical parameters may have better accuracy than the result of a salivary test alone in order to relate the pathologic process with the clinical picture of this disease where saliva would be the most logical sample for correlation.
10. Important consideration would be proper testing and handling of mucoid plugs since it was found in a considerable number of patients specially those who are with longer disease duration, at risk or already developed MALT-L, these plugs would provide valuable information once analysed since they are not normally secreted by parotid glands.

6.3 Conclusion

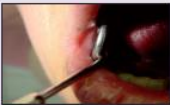









In conclusion, it is known that SS is a slowly progressing disease and the present follow-up study has shown that all of the clinical parameters remained relatively the same over a period of time in patients with longer disease duration. The data implicate a greater role of these clinical parameters in differentiating SS subgroups from other dry mouth patients especially the advanced SS subgroups (at risk and who developed MALT-I). Furthermore, it can be concluded that USS would be the ideal non-invasive test to discriminate and monitor SS patients in general especially in the advanced subgroups.

The aim of developing salivary diagnostics remains an important goal which offers a number of potential applications. The present study evaluated the use of some potential salivary biomarkers (cytokines and S100A8/A9). This study supports previous reports indicating the value of saliva as a diagnostic tool in Sjögren's syndrome. Salivary levels of S100A8/A9 reflect more local inflammatory activity showing the greatest difference between groups in parotid saliva. Salivary cytokine levels were not clear as with the S100A8/A9 levels, no pattern could be drawn from the changes in cytokine levels. However, levels of IL-6 and -4 in parotid saliva continued to show significant differences using different immune-based assays. The levels of cytokines tend to show more prominent differences in whole mouth saliva due to the contribution from their other sources. Further work is required particularly to improve the sensitivity of their diagnostic applications and to explore further combining these results with the clinical parameters that are part of the routine clinical assessment of SS.

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Appendix 1: The Challacombe Scale

<h1>The Challacombe Scale</h1> <p>of Clinical Oral Dryness</p> <p>The Challacombe Scale was developed from research conducted at King's College London Dental Institute under the supervision of Professor Stephen Challacombe*. The purpose of this scale is to be able to visually identify and quantify whether your patient has xerostomia (dry mouth) and if so, how it changes over time and the most appropriate therapy options. This scale is applicable whatever your profession.</p> <p>The Challacombe Scale works as an additive score of 1 to 10 : 1 being the least and 10 being the most severe. Each feature scores 1 and symptoms will not necessarily progress in the order shown, but summated scores indicate likely patient needs. Score changes over time can be used to monitor symptom progression or regression.</p>		
1		Mirror sticks to buccal mucosa
2		Mirror sticks to tongue
3		Saliva frothy
4		No saliva pooling in floor of mouth
5		Tongue shows generalised shortened papillae (mild depapillation)
6		Altered gingival architecture (ie. smooth)
7		Glassy appearance of oral mucosa, especially palate
8		Tongue lobulated / fissured
9		Cervical caries (more than two teeth)
10		Debris on palate or sticking to teeth

Clinical oral dryness scale to derive CODS assessed as mild, moderate or severe (http://www.dentalhealth.org/uploads/download/resourcefiles/download_68_1_The%20Challacombe%20Scale.pdf)

Appendix 2: Sjögren's Ultrasound Score template

Sjögren's Ultrasound reporting template (May 2012)

Scoring system (based on Hocevar 2005)

1. Echogenicity

- Normal = 0
- hypoechoic = 1

2. Consistency / degree of honeycomb/multifocal heterogeneity

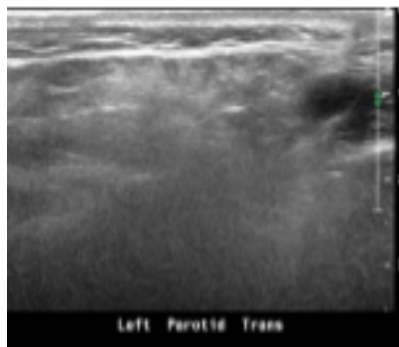
- normal = 0
- mild honeycomb heterogeneity = 1
- evident honeycomb heterogeneity = 2
- gross honeycomb heterogeneity = 3

3. Glands involved

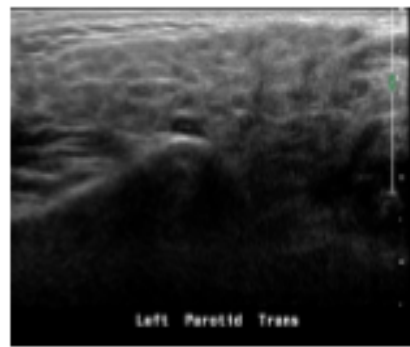
- no gland or only a single gland involvement = 0
- parotids or submandibular glands = 1
- all parotid and submandibular glands involved = 2

4. Hypoechoic foci

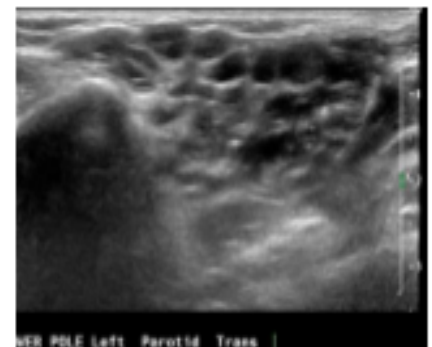
- no distinct enlarged foci = 0
- small distinct dark foci (2-5mm) = 1
- large distinct non-vascular foci (5-8mm) = 2
- larger (>8mm) and/or vascular foci = 3



Mild
heterogeneity



Evident
heterogeneity



Gross
heterogeneity

Appendix 3: American-European Consensus Group (AECG) criteria

Revised international classification criteria for Sjögren's syndrome

I. **Ocular symptoms:** a positive response to at least one of the following questions:

- Have you had daily, persistent, troublesome dry eyes for more than 3 months?
- Do you have a recurrent sensation of sand or gravel in the eyes?
- Do you use tear substitutes more than 3 times a day?

II. **Oral symptoms:** a positive response to at least one of the following questions:

- Have you had a daily feeling of dry mouth for more than 3 months?
- Have you had recurrently or persistently swollen salivary glands as an adult?
- Do you frequently drink liquids to aid in swallowing dry food?

III. **Ocular signs:** that is, objective evidence of ocular involvement defined as a positive result for at least one of the following two tests:

- Schirmer's I test, performed without anaesthesia (<5 mm in 5 minutes)
- Rose bengal score or other ocular dye score (>4 according to van Bijsterveld's scoring system)

IV. **Histopathology:** In minor salivary glands (obtained through normal-appearing mucosa) focal lymphocytic sialadenitis, evaluated by an expert

- histopathologist, with a focus score >1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm² of glandular tissue

V. **Salivary gland involvement:** objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic tests:

- Unstimulated whole salivary flow (<1.5 ml in 15 minutes)
- Parotid sialography showing the presence of diffuse sialectasias (punctate, cavitary or destructive pattern), without evidence of obstruction in the major ducts
- Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer.

VI. **Autoantibodies:** presence in the serum of the following autoantibodies:

- Antibodies to Ro(SSA) or La(SSB) antigens, or both

Appendix 3 (continue)

Revised rules for classification

For primary SS

In patients without any potentially associated disease, primary SS may be defined as follows:

- a. The presence of any 4 of the 6 items is indicative of primary SS, as long as either item IV (Histopathology) or VI (Serology) is positive
- b. The presence of any 3 of the 4 objective criteria items (that is, items III, IV, V, VI)
- c. The classification tree procedure represents a valid alternative method for classification, although it should be more properly used in clinical-epidemiological survey

For secondary SS

In patients with a potentially associated disease (for instance, another well-defined connective tissue disease), the presence of item I or item II plus any 2 from among items III, IV, and V may be considered as indicative of secondary SS

(Vitali et al., 2002)

Appendix 4: Unstimulated Whole Saliva Flow Rate and Collection

Materials:

Balance

Pre-weighed and labelled 20 ml tubes and lids

Clock or timer

1. The unstimulated whole saliva collection procedure should be done before the stimulated parotid collection procedure.
2. During the collection period, the subject shall be seated straight up with eyes open and head tilted slightly forward.
3. The subject will be instructed to minimize oro-facial movements to minimize influence on salivary flow (the subject should not swallow and should not speak during the collection process).
4. Then the patient allows the saliva to accumulate in the floor of the mouth for 60 seconds without swallowing.
5. The patient empties the entire accumulated saliva into the pre-weighed container for 10 minutes.
6. The collection vial is weighed both before and after collection, and both pre- and post-collection weights are recorded.
7. Calculate flow rate on a ml/minute basis.

For detailed information please follow the link provided below

(<https://sicca-online.ucsf.edu/documents/Oral-Saliva-SOP.pdf>)

Appendix 5: Stimulated Parotid Flow Rate and Collection

Materials:

Pre-weighed and labelled 20 ml tubes and lids

2% Citric acid solution (2 g in 100 ml sterile water, kept refrigerated) (2 ml)

Parotid saliva collector (lashley cup)

Tygon tubing (10.2 cm for the collecting tube from the inner circle of the parotid saliva collector, and 30.5-40.6 cm to connect the parotid collector to syringe)

10 ml syringe

Medium Binder Clip (clamp)

Ice and container

Timer or clock

Balance

1. The orifice of the parotid duct is located on the buccal mucosa opposite the upper second molar tooth; dry the area with gauze for better vision.
2. The parotid collector is placed on the mucosa so that the inner ring surrounds the duct orifice.
3. The collector is held on the mucosa by suction from the outer ring, created by pulling back on the syringe and allowing the pressure to come to equilibrium.
 - a) Syringe can then be rested on the patient's shoulder.
 - b) A medium binder clip is attached to the tygon tubing going from the collector to the syringe to "lock-in" the air in the tubing. The suction created should be sufficient to hold the cup in place without occluding the inner chamber of the parotid collector with tissue (i.e., not too much suction).
4. Saliva from the parotid gland then flows passively into the inner ring and through the attached tubing into pre-weighed tube.
5. 2% citric acid solution is applied to the posterior lateral surfaces of the tongue, bilaterally, using 2 drops every 30 seconds to stimulate secretion.
6. Flow may not begin for a minute or two after stimulation has been applied.
7. A maximum of 5 minutes is allowed for saliva to appear in the clear portion of the tubing.
8. If saliva flow is observed during this 5-minute period, an additional 10 minutes is allowed for the saliva to reach the end of the tubing and the 10-minute collection period begins when saliva begins to exit the Tygon tube.
9. Calculate flow rate on a ml/minute basis.

For detailed information please follow the link provided below

(<https://sicca-online.ucsf.edu/documents/Oral-Saliva-SOP.pdf>)

Appendix 6: Human S100A8/S100A9 Heterodimer Quantikine ELISA protocol:

(cat. no. DS8900)

All reagents and samples were brought to room temperature and prepared as directed in chapter 2 subsection 2.2.5.

Fifty μL of Assay Diluent RD1-34 was added to each well. Then 50 μL of Standard and sample is added in duplicates and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.

After washing (four times), 200 μL of Human S100A8/S100A9 Heterodimer Conjugate is added and incubated for another 2 hours at room temperature on the shaker.

Subsequently another wash was done and 200 μL of Substrate Solution is added and incubated for 30 minutes at room temperature on the benchtop protecting it from light.

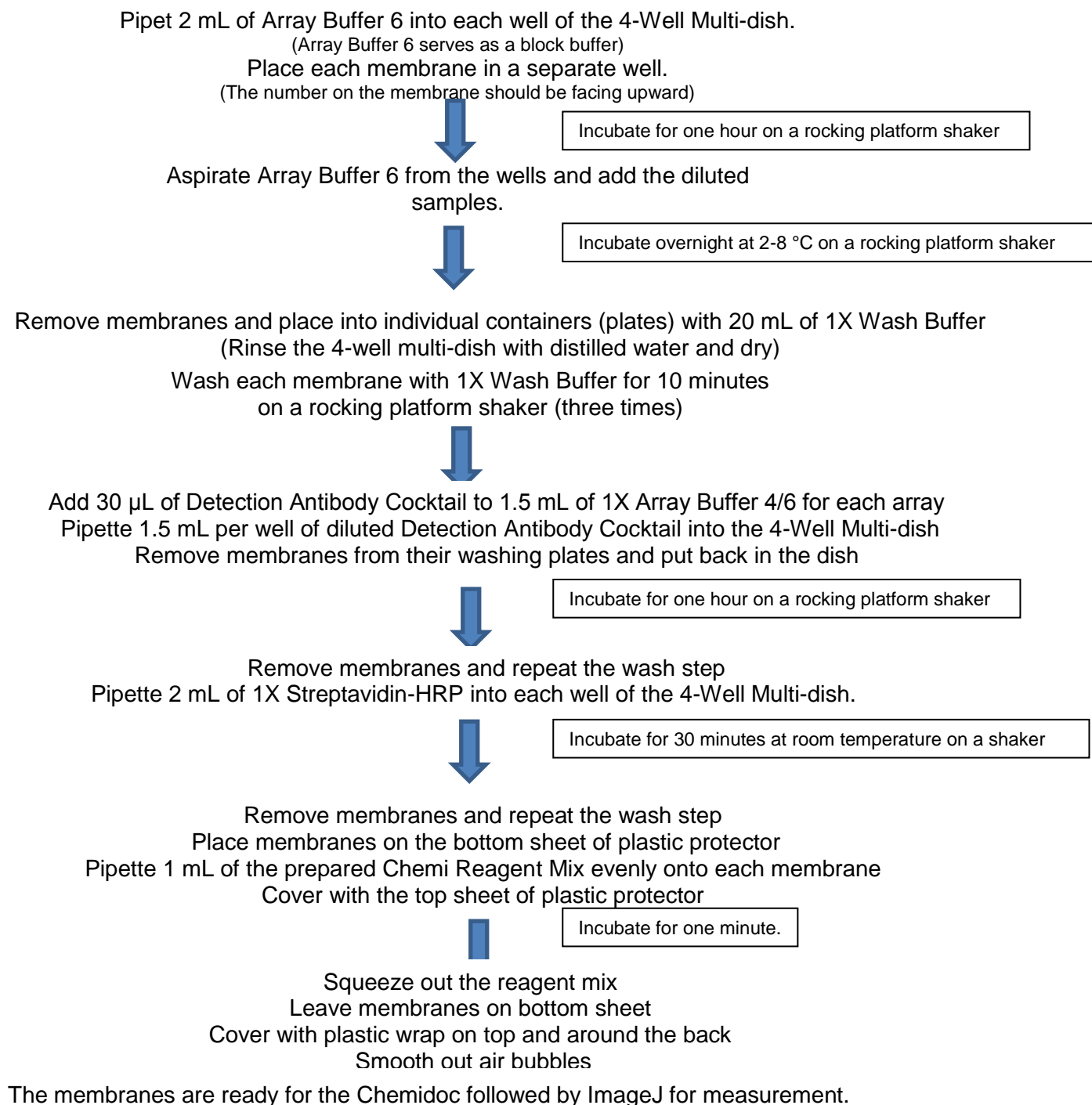
The colour is stopped by the addition of 50 μL of Stop Solution to each well.

The optical density is determined using a microplate reader set to 540 nm at first and subtracted from reading set at 450. This subtraction will correct for optical imperfections in the plate.

Appendix 7: Proteome Profiler Human XL Cytokine Array protocol:

(cat. no. ARY022)

All reagents and samples were brought to room temperature and prepared as directed in chapter 2 subsection 2.2.6. A summary of the protocol is provided below



Appendix 8: Luminex Human Magnetic Assay protocols:

Screening Human Magnetic Assay protocol:

(Premixed multi-analyte kit) (cat. no. LXSAHM)

Microparticle Cocktail (beads) preparation is done by vial centrifugation, vortexing then diluting (250 µL of the cocktail + 2.5 mL Diluent RD2-1). Biotin antibody cocktail is treated similarly. Streptavidin-PE is diluted with wash buffer (110 µL + 2.7 mL).

After sample /standards /reagents preparation the beads are re-suspended before adding them to the wells (25 µL). Samples and standards are then added (25 µL) and incubated on shaker at room temperature for two hours. The wells are washed using magnetic device intended to accommodate the plate (three times). Diluted antibody cocktail is then added (25 µL) and incubated for one hour on the shaker. Wash step is repeated and followed by the addition of diluted streptavidin-PE (25 µL) with an incubation period of 30 minutes. Wash step is repeated and the beads are re-suspended by adding 100 µL of wash buffer to each well and incubated for two minutes on the shaker. The plate is now ready to read.

Performance Human Magnetic Assay protocol:

(Cytokine kit A) (cat. no. FCSTM03)

In general, it is the same as the screening assay with different volumes, diluents (more sensitive) and incubation time. Bead preparation is mentioned before; the dilution is (12.5 µL from each microparticle vial + 2.5 mL microparticle diluent). Biotin antibody cocktail is prepared likewise with its relevant diluent. Streptavidin-PE is diluted with wash buffer (30 µL + 3 mL). The first incubation period of beads and samples/standards is three hours. The following steps are the same as before.

Appendix 9: Preliminary proteins identified by LC-MS/MS proteomics analysis and selected per fold change ratio (first run)

NO	Selected proteins	Total ion current (TIC)			Fold change ratio		
		CT	SS	SS at risk	SS/CT	SS at risk/ CT	SS at risk/ SS
1	Annexin A1	15860.7	73230	1513363.2	4.6	95.4	20.7
2	<u>Actin, cytoplasmic 2</u>	93043.9	1580633.9	3826055.5	17	41.1	2.4
3	<u>Ig γ-1 chain C region</u>	335437.5	2676308.9	13318238.8	8	39.7	5
4	<u>Protein S100-A8</u>	26224.7	299185.7	712901.1	11.4	27.2	2.4
5	Lactotransferrin	18742385.1	7150834.6	69818117	0.4	3.7	9.8
6	<u>Thrombospondin-1</u>	112670	723090	2951800	6.4	26	4.1
7	Thioredoxin	ND	221771.9	139336.5	*	*	0.6
8	<u>14-3-3 protein zeta/delta</u>	ND	392907.5	384322	*	*	1
9	<u>Protein S100-A9</u>	ND	616106.3	1301008.8	*	*	2.1
10	Golgi apparatus protein 1	ND	ND	156208.9	*	*	*
11	Matrilysin	ND	ND	141751.1	*	*	*

CT; healthy control, SS; Sjögren's syndrome, SS at risk of lymphoma of developing mucosa-associated lymphoid tissue. Proteins were present in all groups but up-regulated in disease sample, not present in control sample or not present in control and SS samples. ND; not detected, *could not be calculated. Highlighted are the common up-regulated proteins when SS was compared to control (n=6). Underlined are the final proteins from which the selected proteins were further analysed via ELISA.

Appendix 10: Preliminary proteins identified by LC-MS/MS proteomics analysis and selected per fold change ratio (second run)

NO	Selected proteins	Total ion current (TIC)			Fold change ratio		
		CT	SS	SS at risk	SS/CT	SS at risk / CT	SS at risk/ SS
1	Annexin A1	10026	9689.43	1180842.4	1	117. 8	121.9
2	<u>Actin cytoplasmic 2</u>	210492.7	878628	850867.97	4.2	4.04	1
3	<u>Ig γ-1 chain C region</u>	211505.2	7948508.2	7981574.9	37.6	37.7	1.004
4	<u>Protein S100-A8</u>	14250.3	119945.89	1149673.8	8.4	80. 7	9.6
5	Lactotransferrin	12328407.3	90109906.93	47019126.6	7.3	3.8	0.5
6	<u>Protein S100-A9</u>	89449.2	363782	4414986.4	4.07	49.4	12.1
7	Thioredoxin	43669.5	58756.5	511520.5	1.3	11.7	8.7
8	14-3-3 protein zeta/delta	ND	11870.75	518438.4	*	*	43.7
9	Thrombospondin-1	ND	1572522.32	462161	*	*	0.3
10	Golgi apparatus protein	ND	89355.6	38873.2	*	*	0.4
11	Matrilysin	ND	268531.1	85661.4	*	*	0.3

CT; healthy control, SS; Sjögren's syndrome, SS at risk of developing lymphoma of mucosa-associated lymphoid tissue. Proteins were present in all groups but up-regulated in disease sample, not present in control sample or not present in control and SS samples. ND; not detected, *could not be calculated. Highlighted are the common up-regulated proteins when SS was compared to control (n=6). Underlined are the final proteins from which the selected proteins were further analysed via ELISA.

Appendix 11: Statistical (SPSS) analysis of S100A8/A9 (Calprotectin, CalpP) levels in parotid saliva (overall SS and control groups)

Case Processing Summary

		Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
GP11							
CalpP	CT	18	100.0%	0	0.0%	18	100.0%
	SNOX	14	100.0%	0	0.0%	14	100.0%
	SS	51	100.0%	0	0.0%	51	100.0%

Tests of Normality

		Kolmogorov-Smirnov ^a			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
GP11							
CalpP	CT	.347	18	.000	.585	18	.000
	SNOX	.321	14	.000	.661	14	.000
	SS	.303	51	.000	.741	51	.000

Descriptives

GP11			Statistic	Std. Error
CalpP	CT	Mean	251.3780	117.22698
		95% Lower Confidence Interval for Mean	4.0507	
		Upper Bound	498.7053	
		5% Trimmed Mean	174.4082	
		Median	31.8955	
		Variance	247358.954	
		Std. Deviation	497.35194	
		Minimum	0.00	
		Maximum	1888.21	
		Range	1888.21	
		Interquartile Range	273.19	
		Skewness	2.600	.536
		Kurtosis	6.937	1.038
	SNOX	Mean	204.2639	74.24008
		95% Lower Confidence Interval for Mean	43.8780	
		Upper Bound	364.6499	
		5% Trimmed Mean	167.1028	
		Median	208.9451	
		Variance	77162.253	
		Std. Deviation	277.78094	
		Minimum	0.00	
		Maximum	1077.43	
		Range	1077.43	
		Interquartile Range	265.30	
		Skewness	2.605	.597
		Kurtosis	8.292	1.154
SS		Mean	1968.8121	371.62781
		95% Lower Confidence Interval for Mean	1222.3757	
		Upper Bound	2715.2485	
		5% Trimmed Mean	1721.5018	
		Median	743.1429	
		Variance	#####	
		Std. Deviation	2653.95340	
		Minimum	0.00	
		Maximum	8948.86	
		Range	8948.86	
		Interquartile Range	3435.00	
		Skewness	1.337	.333
		Kurtosis	.416	.656

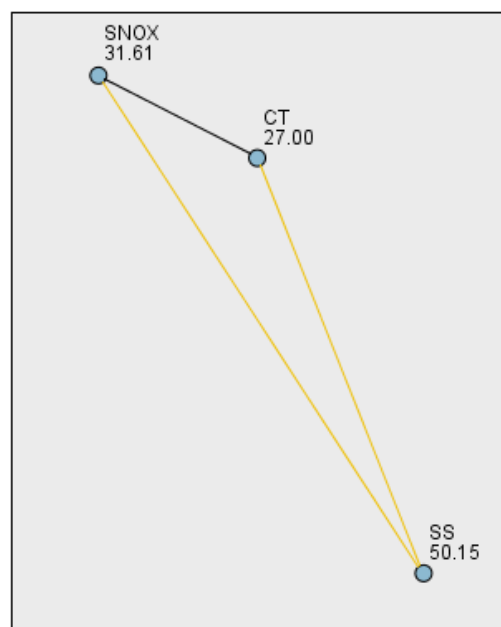
Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of CalpP is the same across categories of GP11.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

GP; groups

Pairwise Comparisons of GP11



Each node shows the sample average rank of GP11.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
CT-SNOX	-4.607	8.529	-.540	.589	1.000
CT-SS	-23.147	6.562	-3.527	.000	.001
SNOX-SS	-18.540	7.222	-2.567	.010	.031

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Appendix 12: Statistical (SPSS) analysis of S100A8/A9 (Calprotectin, CalpP) levels in parotid saliva (SS sub groups and control groups)

Case Processing Summary

		Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
GP22	CalpP						
	CT	18	100.0%	0	0.0%	18	100.0%
	SNOX	14	100.0%	0	0.0%	14	100.0%
	SS	19	100.0%	0	0.0%	19	100.0%
	AT RISK	18	100.0%	0	0.0%	18	100.0%
	5.00	14	100.0%	0	0.0%	14	100.0%

Tests of Normality

		Kolmogorov-Smirnov ^a			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
GP22	CalpP						
	CT	.347	18	.000	.585	18	.000
	SNOX	.321	14	.000	.661	14	.000
	SS	.318	19	.000	.647	19	.000
	AT RISK	.290	18	.000	.764	18	.000
	5.00	.262	14	.010	.825	14	.010

a. Lilliefors Significance Correction

Descriptives

GP22			Statistic	Std. Error
CalpP	CT	Mean	251.3780	117.22698
		95% Confidence Interval for Mean	4.0507	
		Lower Bound		
		Upper Bound	498.7053	
		5% Trimmed Mean	174.4082	
		Median	31.8955	
		Variance	247358.954	
		Std. Deviation	497.35194	
		Minimum	0.00	
		Maximum	1888.21	
		Range	1888.21	
		Interquartile Range	273.19	
		Skewness	2.600	.536
		Kurtosis	6.937	1.038
	SNOX	Mean	204.2639	74.24008
		95% Confidence Interval for Mean	43.8780	
		Lower Bound		
		Upper Bound	364.6499	
		5% Trimmed Mean	167.1028	
		Median	208.9451	
		Variance	77162.253	
		Std. Deviation	277.78094	
		Minimum	0.00	
		Maximum	1077.43	
		Range	1077.43	
		Interquartile Range	265.30	
		Skewness	2.605	.597
		Kurtosis	8.292	1.154

SS	Mean		1163.8734	431.36932
	95% Confidence Interval for Mean	Lower Bound	257.6001	
		Upper Bound	2070.1467	
	5% Trimmed Mean		871.7872	
	Median		506.0000	
	Variance		3535510.254	
	Std. Deviation		1880.29526	
	Minimum		0.00	
	Maximum		7585.30	
	Range		7585.30	
	Interquartile Range		946.86	
	Skewness		2.611	.524
	Kurtosis		7.466	1.014
AT RISK	Mean		2432.5382	728.53053
	95% Confidence Interval for Mean	Lower Bound	895.4732	
		Upper Bound	3969.6033	
	5% Trimmed Mean		2205.6615	
	Median		666.8104	
	Variance		9553621.187	
	Std. Deviation		3090.89327	
	Minimum		0.00	
	Maximum		8948.86	
	Range		8948.86	
	Interquartile Range		5511.19	
	Skewness		.989	.536
	Kurtosis		-.654	1.038
MALT	Mean		2465.0097	764.94043
	95% Confidence Interval for Mean	Lower Bound	812.4564	
		Upper Bound	4117.5631	
	5% Trimmed Mean		2279.3600	
	Median		937.4286	
	Variance		8191873.956	
	Std. Deviation		2862.14499	
	Minimum		0.00	
	Maximum		8271.71	
	Range		8271.71	
	Interquartile Range		4476.00	

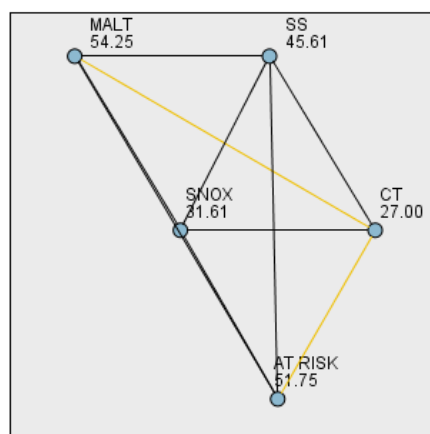
Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of CalpP is the same across categories of GP22.	Independent-Samples Kruskal-Wallis Test	.002	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

GP; groups

Pairwise Comparisons of GP22

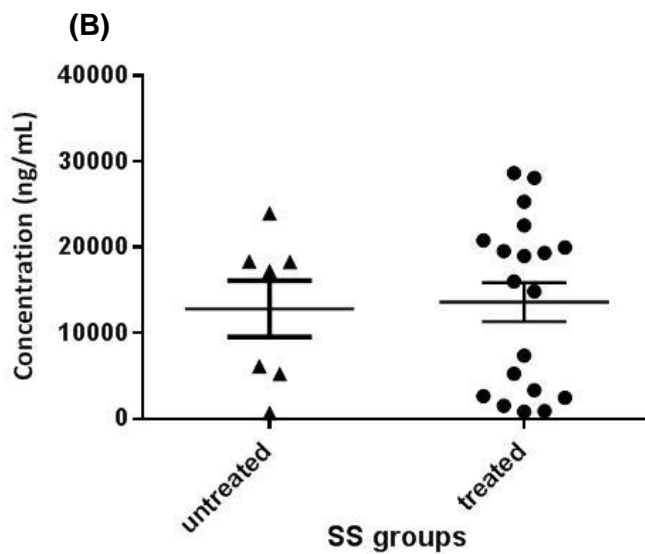
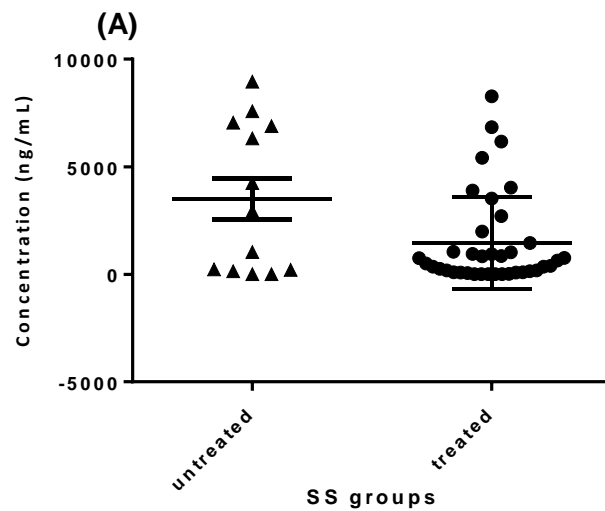


Each node shows the sample average rank of GP22.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
CT-SNOX	-4.607	8.529	-.540	.589	1.000
CT-SS	-18.605	7.873	-2.363	.018	.181
CT-AT RISK	-24.750	7.978	-3.102	.002	.019
CT-MALT	-27.250	8.529	-3.195	.001	.014
SNOX-SS	-13.998	8.430	-1.660	.097	.968
SNOX-AT RISK	-20.143	8.529	-2.362	.018	.182
SNOX-MALT	-22.643	9.047	-2.503	.012	.123
SS-AT RISK	-6.145	7.873	-.781	.435	1.000
SS-MALT	-8.645	8.430	-1.025	.305	1.000
AT RISK-MALT	-2.500	8.529	-.293	.769	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Appendix 13: S100A8/A9 levels in (A) parotid and (B) whole mouth saliva of SS treated and untreated groups



No significant differences were detected between treated (whole/parotid) (n=19/38) and untreated (whole/parotid) (n=7/13) patients (Mann–Whitney U test).

Appendix 14: Summary of the second ranked cytokines-proteins ordered by fold change and p-value when compared to controls (proteome profiler)

		SS vs. CT		MALT-L risk vs. CT		MALT-L vs. CT	
Analytes (23/37)		Fold change	p-value	Fold change	p-value	Fold change	P-value
(A)	MIP-3 α	2.4	NS	1.8	NS	6.4	NS
	TFF3	2.9	NS	2.9	NS	3.5	NS
(B)	TNF-alpha	>-1.5, <1.5	NS	1.7	NS	3.1	NS
	Pentraxin-3	>-1.5, <1.5	NS	1.6	NS	2.7	NS
	SDF-1 α	>-1.5, <1.5	NS	1.6	NS	2.1	NS
	TGF- α	>-1.5, <1.5	NS	1.6	NS	1.6	NS
	MIP-3 β	>-1.5, <1.5	NS	1.6	NS	5.5	NS
	IL-2	>-1.5, <1.5	NS	1.5	NS	1.6	NS
	I-TAC	>-1.5, <1.5	NS	2	NS	4.6	NS
(C)	IL-27	-1.6	NS	1.7	NS	>-1.5, <1.5	NS
	IL-32 $\alpha/\beta/\gamma$	-2	NS	1.7	NS	>-1.5, <1.5	NS
	MCP-1	>-1.5, <1.5	NS	2.1	0.04*	>-1.5, <1.5	NS
	IL-23	>-1.5, <1.5	NS	2.1	NS	>-1.5, <1.5	NS
	IL-24	>-1.5, <1.5	NS	>-1.5, <1.5	NS	1.5	NS
	GRO- α	>-1.5, <1.5	NS	>-1.5, <1.5	NS	4.2	NS
	Chitinase 3-like 1	>-1.5, <1.5	NS	>-1.5, <1.5	NS	3.6	NS
	MIP-1 α /MIP-1 β	>-1.5, <1.5	NS	>-1.5, <1.5	NS	2.3	NS
	FGF basic	>-1.5, <1.5	NS	>-1.5, <1.5	NS	1.7	NS
	BAFF	>-1.5, <1.5	NS	>-1.5, <1.5	NS	1.5	NS
	C-Reactive protein	>-1.5, <1.5	NS	>-1.5, <1.5	NS	2.9	NS
	G-CSF	-2	NS	>-1.5, <1.5	NS	1.8	NS
	Complement C5/C5a	-1.9	NS	>-1.5, <1.5	NS	2.3	NS
	ICAM-1	-1.6	NS	>-1.5, <1.5	NS	3.4	NS

Fold change in the first group (A) is ≥ 1.5 in all disease groups. In the second group (B) it is increased in 2 of the disease groups. While the third group (C) fold change is increased in one group only. NS; not significant, CT; healthy controls, SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma. MCP-1 is significantly different when compared to controls (one- way ANOVA followed by Tukey HSD post hoc test).

Second ranked proteins were identified in 11 samples of 12

Appendix 15: Summary of the second ranked cytokines-proteins ordered by fold change and p-value when compared to SS (proteome profiler)

		MALT-L risk vs. SS		MALT-L vs. SS		MALT-L vs. MALT-L risk	
Analytes (34/37)		Fold change	p-value	Fold change	p-value	Fold change	P-value
(A)							
ICAM-1		2.7	NS	5.1	NS	2.4	NS
I-TAC		2	NS	4.6	NS	2.6	NS
MIP-3 β		1.5	NS	5.4	NS	4	NS
C-Reactive protein		2.4	NS	4	NS	1.6	NS
Complement C5/C5a		2	NS	4.1	NS	2.1	NS
(B)							
IL-27		3.3	NS	1.7	NS	-1.6	NS
IL-32 $\alpha/\beta/\gamma$		3.6	0.02*	2	NS	-1.7	NS
MIP-1 α /MIP-1 β		2.6	NS	3.6	NS	>-1.5, <1.5	NS
Dkk-1		3	NS	3.3	NS	>-1.5, <1.5	NS
BDNF		2.5	NS	3.3	NS	>-1.5, <1.5	NS
Angiopoietin-2		2.8	NS	3.1	NS	>-1.5, <1.5	NS
TNF-alpha		1.5	NS	3	NS	>-1.5, <1.5	NS
FGF basic		2.2	NS	2.5	NS	>-1.5, <1.5	NS
IL-15		2.2	NS	2.5	NS	>-1.5, <1.5	NS
IL-34		2	NS	2.2	NS	>-1.5, <1.5	NS
BAFF		1.5	NS	2	NS	>-1.5, <1.5	NS
IL-24		2.6	NS	2.8	NS	>-1.5, <1.5	NS
G-CSF		3.4	NS	3.9	NS	>-1.5, <1.5	NS
TARC		2.3	NS	2.1	NS	>-1.5, <1.5	NS
Cripto-1		2.9	NS	2.6	NS	>-1.5, <1.5	NS
TGF- α		2.3	NS	2.3	NS	>-1.5, <1.5	NS
IL-12 p70		2.9	NS	2	NS	>-1.5, <1.5	NS
GRO- α		>-1.5, <1.5	NS	5.6	NS	4.3	NS
MIP-3 α		>-1.5, <1.5	NS	4	NS	4.6	NS
SDF-1 α		>-1.5, <1.5	NS	1.6	NS	2.1	NS
Chitinase 3-like 1		>-1.5, <1.5	NS	4.2	NS	3.5	NS

Appendix 15 (Continued)

Analytes	MALT-L risk vs. SS		MALT-L vs. SS		MALT-L vs. MALT-L risk	
	Fold change	p-value	Fold change	p-value	Fold change	P-value
(C) MCP-1	2.2	0.03*	>-1.5, <1.5	NS	-2.2	NS
IL-10	2.2	0.04*	>-1.5, <1.5	NS	>-1.5, <1.5	NS
IL-13	2.1	NS	>-1.5, <1.5	NS	>-1.5, <1.5	NS
MCP-3	2.1	0.05*	>-1.5, <1.5	NS	>-1.5, <1.5	NS
RANTES	1.9	NS	>-1.5, <1.5	NS	>-1.5, <1.5	NS
PDGF-AB/BB	1.8	NS	>-1.5, <1.5	NS	>-1.5, <1.5	NS
Pentraxin-3	>-1.5, <1.5	NS	2.5	NS	>-1.5, <1.5	NS
IL-2	>-1.5, <1.5	NS	1.5	NS	>-1.5, <1.5	NS

Fold change in the first group (A) is ≥ 1.5 in all disease groups. In the second group (B) it is increased in 2 of the disease groups. While the third group (C) fold change is increased in one group only. NS; not significant, CT; healthy controls, SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma. IL-32 $\alpha/\beta/\gamma$, MCP-1, IL-10 and MCP-3 are significantly different when compared to SS (one- way ANOVA followed by Tukey HSD post hoc test).

Second ranked proteins were identified in 11 samples of 12

Appendix 16: Summary of the third ranked cytokines-proteins ordered by fold change and p-value when compared to controls (proteome profiler)

		SS vs. CT		MALT-L risk vs. CT		MALT-L vs. CT	
Analytes (8/20)		Fold change	p-value	Fold change	p-value	Fold change	p-value
(A)							
IL-3		-1.5	NS	1.8	NS	2.4	NS
MIF		>-1.5, <1.5	NS	1.8	NS	2.4	NS
(B)							
Angiopoietin-1		>-1.5, <1.5	NS	>-1.5, <1.5	NS	2.4	NS
IL-5		-3.2	NS	>-1.5, <1.5	NS	2	NS
IGFBP-2		-1.6	NS	>-1.5, <1.5	NS	4.7	NS
IL-8		-1.6	NS	>-1.5, <1.5	NS	4.9	NS
Aggrecan		>-1.5, <1.5	NS	>-1.5, <1.5	NS	1.9	NS
FGF-7		-1.6	NS	1.8	NS	>-1.5, <1.5	NS

Fold change in the first group (A) is ≥ 1.5 in 2 of the disease groups. While the second group (B) fold change is increased in one group only. NS; not significant, CT; healthy controls, SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma. No significant differences detected when compared to controls (one- way ANOVA followed by Tukey HSD post hoc test).

Third ranked proteins were identified in 10 samples or less of 12

Appendix 17: Summary of the third ranked cytokines-proteins ordered by fold change and p-value when compared to SS (proteome profiler)

	MALT-L risk vs. SS		MALT-L vs. SS		MALT-L vs. MALT-L risk	
Analytes (16/20)	Fold change	p- value	Fold change	p- value	Fold change	P- value
M-CSF	2.2	NS	2.8	NS	>-1.5, <1.5	NS
MIF	3.2	NS	3.8	NS	>-1.5, <1.5	NS
IL-3	3.3	NS	3.9	NS	>-1.5, <1.5	NS
IL-5	4.1	NS	5.1	NS	>-1.5, <1.5	NS
IGFBP-3	1.8	NS	1.6	NS	>-1.5, <1.5	NS
IL-1β	1.9	NS	1.6	NS	>-1.5, <1.5	NS
PF4	2.6	NS	1.7	NS	>-1.5, <1.5	NS
RAGE	2.7	NS	2.2	NS	>-1.5, <1.5	NS
IL-31	3.4	NS	2.7	NS	>-1.5, <1.5	NS
FGF-7	3.3	NS	2.8	NS	>-1.5, <1.5	NS
IL-1α	3.8	NS	2.9	NS	>-1.5, <1.5	NS
Angiopoietin-1	1.5	NS	3	NS	>-1.5, <1.5	NS
Aggrecan	1.9	NS	3.2	NS	>-1.5, <1.5	NS
Growth hormone	5.6	NS	5.1	NS	>-1.5, <1.5	NS
Leptin	1.8	NS	>-1.5,<1.5	NS	>-1.5, <1.5	NS
IFN-γ	1.9	NS	>-1.5,<1.5	NS	>-1.5, <1.5	NS

Fold change in the first group (A) is ≥ 1.5 in 2 of the disease groups. While the second group (B) fold change is increased in one group only. NS; not significant, CT; healthy controls, SS; Sjögren's syndrome, MALT-L; mucosa associated lymphoid tissue lymphoma. No significant differences detected when compared to SS (one- way ANOVA followed by Tukey HSD post hoc test).

Third ranked proteins were identified in 10 samples or less of 12

Appendix 18: Statistical SPSS analysis of IL-6 levels in parotid saliva (SS sub groups)

Case Processing Summary

Group2		Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
IL6P	CT	18	100.0%	0	0.0%	18	100.0%
	SNOX	15	100.0%	0	0.0%	15	100.0%
	SS	19	100.0%	0	0.0%	19	100.0%
	AT RISK	17	100.0%	0	0.0%	17	100.0%
	MALT	13	100.0%	0	0.0%	13	100.0%

Descriptives

Group2			Statistic	Std. Error
IL6P	CT	Mean	1.4233	.19230
		95% Lower Confidence Interval for Mean	1.0176	
		Upper Bound	1.8290	
		5% Trimmed Mean	1.4104	
		Median	1.5900	
		Variance	.666	
		Std. Deviation	.81584	
		Minimum	.16	
		Maximum	2.92	
		Range	2.76	
		Interquartile Range	1.40	
		Skewness	-.031	
		Kurtosis	-.706	
		Mean	1.4893	.19165
SNOX		95% Lower Confidence Interval for Mean	1.0783	
		Upper Bound	1.9004	
		5% Trimmed Mean	1.4715	
		Median	1.4000	
		Variance	.551	
		Std. Deviation	.74227	
		Minimum	.34	
		Maximum	2.96	

SS	Range		2.62	
	Interquartile Range		.62	
	Skewness		.199	.580
	Kurtosis		.000	1.121
	Mean		18.2832	7.54027
	95% Confidence Interval for Mean	Lower Bound	2.4416	
		Upper Bound	34.1247	
	5% Trimmed Mean		14.1246	
	Median		2.5400	
	Variance		1080.259	
	Std. Deviation		32.86729	
	Minimum		.63	
	Maximum		110.79	
	Range		110.16	
	Interquartile Range		9.97	
AT RISK	Skewness		2.084	.524
	Kurtosis		3.367	1.014
	Mean		11.4635	3.84078
	95% Confidence Interval for Mean	Lower Bound	3.3214	
		Upper Bound	19.6056	
	5% Trimmed Mean		9.3989	
	Median		4.9200	
	Variance		250.776	
	Std. Deviation		15.83592	
	Minimum		.42	
	Maximum		59.67	
	Range		59.25	
	Interquartile Range		18.77	
	Skewness		2.067	.550
	Kurtosis		4.592	1.063
MALT	Mean		10.2454	4.19193
	95% Confidence Interval for Mean	Lower Bound	1.1120	
		Upper Bound	19.3788	
	5% Trimmed Mean		8.5776	
	Median		2.5400	
	Variance		228.440	
	Std. Deviation		15.11422	

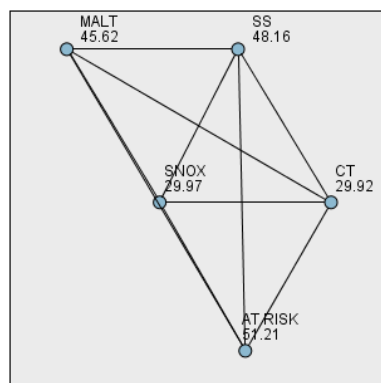
Minimum	.51	
Maximum	50.00	
Range	49.49	
Interquartile Range	15.17	
Skewness	1.929	.616
Kurtosis	3.266	1.191

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of ILBP is the same across categories of Group2.	Independent-Samples Kruskal-Wallis Test	.013	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Pairwise Comparisons of Group2



Each node shows the sample average rank of Group2.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
CT-SNOX	-.050	8.224	-.006	.995	1.000
CT-MALT	-15.708	8.767	-1.792	.073	.732
CT-SS	-18.241	7.737	-2.358	.018	.184
CT-AT RISK	-21.289	7.956	-2.676	.007	.075
SNOX-MALT	-15.658	9.111	-1.719	.086	.857
SNOX-SS	-18.191	8.125	-2.239	.025	.252
SNOX-AT RISK	-21.239	8.333	-2.549	.011	.108
MALT-SS	2.533	8.674	.292	.770	1.000
MALT-AT RISK	5.581	8.869	.629	.529	1.000
SS-AT RISK	-3.048	7.853	-.388	.698	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Appendix 19: Analytes excluded from further validation via Luminex screening assay

Biomarker	Study	Disease	Sample	Method
CD30 (Tumour marker) (1 ST rank)	(Bassig et al., 2015)	NHL(B- cell subtypes)	Serum	ELISA
	(Gerli et al., 1999, Ichikawa et al., 1998)	SS	Serum	
Vitamine D BP (1 ST rank)	(Fleissig et al., 2009)	SS	WMS*	ESI-MS/MS
ENDOGLIN (1 ST rank)	(Schimming and Marme, 2002)	Oral squamous cell carcinoma	Tissue	Immunohisto-chemistry

SS; Sjögren's syndrome, NHL; Non-Hodgkin lymphoma, PS; parotid saliva, WMS; whole mouth saliva, GCF; gingival crevicular fluid, ESI; electrospray ionization, MS/MS; Tandem mass spectrometry, 2DE, two-dimensional gel electrophoresis * down regulated, + the same level as healthy controls

Appendix 20: Cytokines identified previously in serum of SS subjects using different methods

Biomarkers	Study	Disease	Sample	Method	Finding
IL-2, srIL-2, IL-6 and IL-10	(Garcic-Carrasco et al., 2001)	SS	Serum	ELISA	All increased
IL-10	(Anaya et al., 2002)	SS	Serum	ELISA	Increased
BAFF	(Mariette et al., 2003)	SS	Serum	ELISA	Increased
IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-13, IL-15, IL-17, IFN- γ , TNF- α , EGF, VEGF, FGF, G-CSF, GM-CSF, MCP-1, Eotaxin, MIP-1 α , MIP-1 β , RANTES, TNF-RI and TNF-RII	(Szodoray et al., 2004)	SS	Serum	25-plex, bead-based assay	IL-1 β , IL-6, IL-8, IL-12p40, IL-15, TNF- α , EGF, MIP-1 β , MCP-1, Eotaxin, and RANTES were increased
IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-13, IL-15, IL-17, IFN- γ , IFN- α , TNF- α , GM-CSF, MCP-1, Eotaxin, MIP-1 α and MIP-1 β	(Szodoray et al., 2005)	SS \pm GC	Serum	20-plex bead-based assay	IL-1 β , IL-2, IL-6, IL-15, IFN- γ and MIP-1 β were increased in both SS \pm GC
BAFF				ELISA	IL-4, IL-10, GM-CSF, IFN- α , MIP-1 α , Eotaxin and BAFF were increased in SS +GC
BAFF and APRIL	(Jonsson et al., 2005)	SS \pm GC	Serum	ELISA	Increased
BAFF	(Pers et al., 2005b)	SS	Serum	ELISA	Increased
BAFF and β 2 microglobulin	(Gottenberg et al., 2005)	SS	Serum	ELISA	Both were not associated BAFF was associated with ENA but not with systemic manifestation
IL-4, IL-10, and IFN- γ	(Szodoray et al., 2008)	SS	Serum	ELISA	IL-4 not different to CT IL-10 decreased while IFN- γ increased
IL-1 β , IL-1Ra, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40/p70, IL-13, IL-15, IL-17, IFN- α , IFN- γ , TNF- α , MCP-1, MIP-1 α , MIP-1 β , RANTES, Eotaxin, MIG, GM-CSF and IP-10	(Reksten et al., 2009)	SS \pm GC and \pm FS	Serum	25-plex, bead-based assay	IL-1beta, IL-4, IL-12p40, IL-15, IL-17, IL-1RA, MIP-1 α , MIP-1 β , Eotaxin and IFN- α were increased in SS+GC

Appendix 20 (Continued)

Biomarkers	Study	Disease	Sample	Method	Finding
FLT3LG	(Tobon et al., 2010)	SS	Serum	ELISA	Increased
IL-22	(Lavoie et al., 2011)	SS	Serum	Luminex bead-based assay	Increased
FLT3LG	(Tobon et al., 2013)	SS±NHL	Serum	ELISA	Increased levels were associated with history of lymphoma
BAFF and APRIL	(Pollard et al., 2013b)	SS and TTT	Serum	ELISA	Both increased in SS BAFF increased after TTT
IL-1 β , IL-1Ra, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL12p40/p70, IL-13, IL-15, IL-17, IFN- α , IFN- γ , TNF- α , MCP-1, MIP-1 α , MIP-1 β , RANTES, Eotaxin, MIG, GM-CSF and IP-10	(Pollard et al., 2013a)	SS and TTT	Serum	25-plex, bead-based assay	Nearly all of the cytokines were increased in SS except for IL-8, IFN- γ and RANTES TTT affected GM-CSF, IL-1Ra, IL-6, IL-10, IFN- α , TNF- α , MIP-1 β and MIG levels
BAFF	(Quartucci et al., 2013)	SS± NHL	Serum	ELISA	Increased levels were associated with history and at risk of lymphoma and higher disease activity
BAFF	(Gottenberg et al., 2013)	SS+NHL	Serum	ELISA	Increased levels associated with higher disease activity scores and history of lymphoma
IL-17	(Alunno et al., 2015)	SS	Serum	ELISA	Detected in some samples
Eotaxin and BCA-1	(Nocturne et al., 2015)	SS± NHL	Serum	Multiplex assay	BCA-1 increased in NHL and associated with higher disease activity scores
BAFF and IL-17A	(Deng et al., 2016)	SS	Serum	ELISA	Both increased

SS; Sjögren's syndrome, NHL, non- Hodgkin's lymphoma, TTT; rituximab treatment, GC; germinal centers, FS; focus score, ELISA; enzyme-linked immunosorbent assay, ENA; extractable nuclear antibodies (anti-SSA/SSB)

Appendix 21: Cytokines identified previously in serum and salivary glands of SS subjects using different methods

Biomarkers	Study	Disease	Sample	Method	Finding
BAFF	(Groom et al., 2002)	SS	Serum MSG	ELISA IHC	Increased Increased expression
		SS model	SMG*	IHC/ flow cytometry	BAFF (Tg) mice developed SS
IL-18	(Bombardieri et al., 2004)	SS	Serum MSG	ELISA Double IHC	Increased Increased expression
BAFF	(Lavie et al., 2008)	SS	PBMCs	ELISA PCR	Increased No difference in BAFF mRNA levels
IL-23	(Katsifis et al., 2009)	SS	Plasma	ELISA	Increased
IL-2, IL-4, IL-6, IL-12, IL-17, IFN- γ and TGF- β 1			Plasma	Luminex bead-based assay	All increased except IFN- γ
IL-6, IL-17, IL-23 and TGF- β 1			MSG	IHC / PCR	mRNA and protein levels of IL-6, -17A and -23 were increased
IL-17 producing cells	(Kwok et al., 2012)	SS	PBMCs	Flow cytometry	Increased
IL-6, IL-17 and IL-23			PBMCs	ELISA	Increased
IL-17, IL-23 and TLRs			MSG	IHC/ Flow cytometry/PCR	mRNA and protein levels of IL-17A and -23 were increased
IL-17	(Fei et al., 2014)	SS	Serum	ELISA	Increased
IL-17 producing cells			PBMCs	Flow cytometry/ PCR	Increased cells and mRNA levels
			MSG	IHC	Increased expression
IL-18, IL-22 and their receptors	(Ciccia et al., 2015)	SS \pm NHL	PBMCs MSG PGx	Flow cytometry/ PCR IHC /Flow cytometry	Aberrant expression of IL-22R1 linked to IL-18

SS; Sjögren's syndrome, NHL; non- Hodgkin's lymphoma, MSG; minor salivary glands, SMG*; submaxillary glands, PBMCs; peripheral blood mononuclear cells, PGx; parotid glands, ELISA; enzyme-linked immunosorbent assay, IHC; immunohistochemistry, PCR; polymerase chain reaction.

Appendix 22: Cytokines identified previously in salivary glands of SS subjects using different methods

Biomarkers	Study	Disease	Sample	Method	Finding
IL-1 α , IL-1 β , IL-4, IL-6 TNF- α , TNF- β and IFN- γ	(Oxholm et al., 1992)	SS	MSG	IHC	IL-1 β , IL-6 TNF- α and IFN- γ were over-expressed in SS IL-1 α , IL-4 and TNF- β were not detected
IL-2, IL-6, IL-10 and TGF- β	(Ogawa et al., 1995)	SS	MSG	IHC / PCR ELISA	mRNA levels of all cytokines are expressed in mod-severe lesions IL6 and -10 were detected
IL-1 β , IL-2, IL-3, IL-4, IL-6, IL-6R, IL-9, IL-10, IL-12, TNF- α , IFN- γ and TGF- β	(De Vita et al., 1995)	SS+MESA SS \pm NHL	PGx	PCR	mRNA levels of all cytokines were expressed in all groups IL-4 absent in MESA but present in some of SS with NHL
IFN- γ , IL-2, IL-4 and IL-5	(Konttinen et al., 1999)	SS	MSG	IHC PCR	IFN- γ , IL-2 were expressed in both in SS and CT mRNA levels of IFN- γ , IL-2 and IL-4 were expressed in SS and CT
IL-2, IL-4, IL-10, IL-12, IL-18, TGF- β , TNF- α , IFN- γ	(Kolkowski et al., 1999)	SS	MSG	IHC / PCR	mRNA levels of Th1 cytokines were expressed IL-4 was absent IL-10 and TGF- β were expressed in majority
SDF-1 and BCA-1	(Amft et al., 2001)	SS \pm GC	MSG	IHC	BCA-1 was expressed in SS not CT SDF-1 was expressed in both
IP-10, MIG and SDF-1	(Ogawa et al., 2002)	SS	MSG	IHC / PCR- ELISA	mRNA levels of IP-10 and MIG were increased
SDF-1, BCA-1 and SLC	(Salomonsson et al., 2003)	SS \pm GC	MSG	IHC	No association with GC
I-TAC	(Ogawa et al., 2004)	SS	MSG	IHC / PCR- ELISA	Expressed in the presence of IN- γ
BAFF	(Lavie et al., 2004)	SS	MSG	IHC / PCR	Strong expression in SS confirmed by PCR

Appendix 22 (Continued)

Biomarkers	Study	Disease	Sample	Method	Finding
BCA-1 and SLC	(Barone et al., 2005)	SS±GC	MSG	IHC	Both expressed with high focus score
IL-12p70 and IL-18	(Manoussakis et al., 2007)	SS and NHL risk	MSG	Double IHC	Both were expressed in SS not CT
SDF-1, BCA-1 and SLC	(Barone et al., 2008)	SS±MALT-L & LESA	MMSG	Double IHC flow cytometry	SDF-1 and SLC expressed in LESA BCA-1 in MALT-L
MCP-1	(Iwamoto et al., 2010)	SS	MSG	IHC / PCR	mRNA and protein levels were increased
IL-17, IL-22 and IL-23	(Ciccia et al., 2012)	SS	MSG	IHC / PCR	mRNA and protein levels of all were increased
IP-10	(Ruffilli, 2014)	SS	MSG	IHC	Increased expression

MIG; Monokine induced by gamma interferon, I-TAC; inducible T-cell alpha chemoattractant, BCA-1; B cell-attracting chemokine -1, SLC; Secondary lymphoid-tissue chemokine, SDF-1; stromal cell-derived factor 1, SS; Sjögren's syndrome, MESA; myoepithelial sialadenitis, NHL; non- Hodgkin's lymphoma, GC; germinal centers, MALT-L; mucosa associated lymphoid tissue lymphoma, LESA; lymphoepithelial sialadenitis, MSG; minor salivary glands, PGx; parotid glands, MMSG; major and minor salivary glands, IHC; immunohistochemistry, PCR; polymerase chain reaction, ELISA; enzyme-linked immunosorbent assay

Appendix 23: Cytokines identified previously in saliva of SS subjects using different methods

Biomarkers	Study	Disease	Sample	Method	Findings
IL-2 and IL-6	(Rhodus et al., 1998)	SS and TTT	Saliva	ELISA	Both increased but decreased after TTT
IL-6	(Tishler et al., 1999)	SS	Saliva	ELISA	IL-6 increased
			Serum		No differences
IFN- γ , IL-2 and IL-6	(Streckfus et al., 2001)	SS and TTT	Saliva	ELISA	IL-2 and -6 increased but decreased after TTT
IL-6 and bFGF	(Boras et al., 2004)	SS	Saliva	ELISA	Both increased
			Serum		No difference
BAFF	(Pers et al., 2005a)	SS	Saliva	ELISA	Increased
			Serum	ELISA	Increased
75 analytes	(Delaleu et al., 2008)	SS model	Saliva	75-plex, bead-based assay (MAP)	34 detected in saliva
82 analytes			Serum	82-plex, bead-based assay (MAP)	38 detected in serum
IL-17 and IL-23	(Nguyen et al., 2008)	SS model	SMG	IHC /PCR	Both expressed in later stages even mRNA levels
IL-17			Saliva Serum	Bead- based assay (mouse)	low levels detected Detected in early stages
IL-17 and IL-23		SS	MSG	IHC	Both were expressed
IL-17 and IL-6			Saliva Serum	Bead- based assay (human)	IL-6 only increased Both did not differ from CT
MCP-1, MIP-1 α , MIP-1 β , IL-8 and IP-10	(Lee et al., 2010)	SS	Saliva	Luminex bead-based assay	MIP-1 α , MIP-1 β , IL-8 and IP-10 were increased
IL-1, IL-4, IL-6, IL-10, IL-12p40, IL-17, IFN- γ and TNF- α	(Kang et al., 2011)	SS	Saliva	Luminex bead-based assay	All were increased

Appendix 23 (Continued)

Biomarkers	Study	Disease	Sample	Method	Finding
SDF_1α, BCA-1, IP-10, MCP-1, MIP-1α and RANTS	(Hernandez-Molina et al., 2011)	SS	Saliva	Luminex bead-based assay	IP-10 and MCP-1 were increased
IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α, IFN-γ, RANTES, IL-8, MIG and IP-10 (TARC, MDC and MIP-1α)	(Moriyama et al., 2012)	SS	Saliva	CBA (ELISA)	IL-1 β, IL-8, IL-4, IL-6, IL-10 and MDC were increased. IL-4, IL-10 and MDC were higher with higher focus scores
IL-2, IFN-γ, IL-4, IL-5, IL-10, IL-12, IL-17 and TGF-β, IP-10, RANTS, TARC, MDC and MIP-1α			MSG	IHC / PCR	mRNA levels of IL-2, IFN-γ, IL-4, IL-10, IL-17, IP-10, MIP-1α, RANTES, TARC and MDC were increased
			PBMCs	PCR	mRNA levels of IP-10 was increased
IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17A, IFN-γ, and TNF-α	(Ohya et al., 2015)	SS	Saliva	CBA	All were increased
187 analytes	(Delaleu et al., 2015)	SS	Saliva	187-plex, bead-based assay (MAP)DFA and GO- analysis	52 were increased in SS 4- and 6- plex signature included IL-4 and IL-5

SS; Sjögren's syndrome, TTT; pilocarpine treatment, SMG; submandibular glands, MSG; minor salivary glands, PBMCs; peripheral blood mononuclear cells, ELISA; enzyme-linked immunosorbent assay, IHC; immunohistochemistry, PCR; polymerase chain reaction, CBA; cytometric bead assay, MAP; multianalyte profile, DFA; discriminant function analysis, GO; gene ontology- based network analysis.

Appendix 24: Cytokines identified previously in parotid saliva of SS subjects using different methods

Biomarkers	Study	Disease	Sample	Method	Finding
IL-1 α , IL-2, IL-4, IL-5, IL-6, IL-10, IFN- γ , and TNF- α	(Fox et al., 1994)	SS	(P) saliva	ELISA	All were increased in SS except IL-4 and -5 were not detected
			MSG	PCR	mRNA levels of IL-2, -10 and IFN- γ were detected in SS but not IL-4 and -5. TNF- α was detected in SS and normal IL-1 α and -6 were detected in some
IL-6	(Grisius et al., 1997)	SS	(P) saliva	ELISA	IL-6 increased
			Serum		IL-6 increased
IL-10, IL-2, IL-4 and IFN- γ	(Bertorello et al., 2004)	SS	(P) saliva	ELISA	IL-10 increased in most IFN- increased in some IL-2 and -4 are absent

SS; Sjögren's syndrome, (P); parotid saliva, MSG; minor salivary glands, ELISA; enzyme-linked immunosorbent assay, PCR; polymerase chain reaction

Appendix 25: Summary of the review of literature on cytokines

The findings of the above- listed studies for chapter 5 can be summarised as follows; **in serum**, levels of IL-22, -10 were increased in SS patients with a direct correlation with some of the clinical parameters, e.g. autoantibody levels (Anaya et al., 2002, Lavoie et al., 2011). Levels of IL-6 were increased in SS patients when compared to controls (Grisius et al., 1997). While another study found no differences between IL-6 levels of SS and control groups (Tishler et al., 1999). IL-17 and linked cytokines (e.g. IL-6) were found to be up-regulated in SS in comparison to controls (Katsifis et al., 2009, Kwok et al., 2012). In addition to IL-17 (Fei et al., 2014). BAFF was also found highly elevated in SS patients, both of which correlated with different SS phenotypes (Deng et al., 2016). BAFF and APRIL were elevated in SS patients (Pollard et al., 2013b). On the other hand, Alunno et al., (2015) reported detectable levels of IL-17 in 15/50 SS patients and that it was associated with longer disease duration which is a similar finding to Nguyen et al., (2008) where they have analysed levels of IL-17 and -23 in sera, saliva and glands of animal models and SS patients. Serum levels of IL-18 in SS were higher than controls but similar to patients with rheumatoid arthritis (Bombardieri et al., 2004). Also, serum levels of several cytokines were measured by multiplex assays in SS patients identifying potential targets (Pollard et al., 2013a, Szodoray et al., 2005, Szodoray et al., 2004, Reksten et al., 2009, Moriyama et al., 2012). **In minor salivary glands**, protein and mRNA levels of IL-6, -17, 22, -23 and MCP-1 were increased in SS patients (Ciccio et al., 2012, Iwamoto et al., 2010, Katsifis et al., 2009, Kwok et al., 2012, Fei et al., 2014) In addition IL-18 was expressed in SS patient's glands but not in chronic sialadenitis glands (Bombardieri et al., 2004). Th1 cytokines were identified in salivary glands of SS patients while IL-4 was absent and IL-10 was present in the majority of samples (Kolkowski et al., 1999). Similar findings were reported, demonstrating cytokine expression in healthy control samples as well (Konttinen et al., 1999). Expression of some chemokines was reported by Ogawa et al., (2004) and (2002) also the same group has looked at other cytokines as well (Ogawa et al., 1995).

Turning now to the non-Hodgkin lymphoma (NHL) literature, it can be seen that a considerable number of studies were done looking at the cytokine/chemokine levels in SS patients with lymphoma, correlating their levels with the predictive markers of lymphoma, where serum and salivary glands were their main targets;

In **salivary glands** of SS patients with non-Hodgkin lymphoma (NHL), there was an aberrant expression of IL-22R1 which is IL-18 dependent (Ciccia et al., 2015). Several cytokines were detected in all SS stages (SS, SS with myoepithelial sialadenitis (MESA) and SS with NHL) but expression levels of IL-4 were lacking from patients with MESA while present in some salivary glands of SS and SS with NHL (De Vita et al., 1995). Some cytokines were increased more in mod-severe lesion (Ogawa et al., 1995). IL-18 was positively correlated with other predictive markers while IL-12 was negatively correlated (Manoussakis et al., 2007). A number of chemokines (BCA-1 and SLC) were expressed in glands being higher with high focus scores (germinal centres) (Barone et al., 2005, Amft et al., 2001). Also BCA-1 expression was increased in salivary glands of SS patients with MALT-L (Barone et al., 2008). However, Salomonsson et al., (2003) found no correlation between those cytokines and the formation of germinal centres.

In **serum**, higher Fms-related tyrosine kinase 3 ligand (FLT3LG) levels were found in SS group in comparison to controls and they were associated with a history of lymphoma as well as with other predictive markers (Tobon et al., 2010, Tobon et al., 2013). Several studies have focused on BAFF levels in SS, correlating them with serological levels (Pers et al., 2005b, Groom et al., 2002, Mariette et al., 2003, Lavie et al., 2008), with certain predictors of lymphoma development, with higher disease activity and with history of lymphoma (Gottenberg et al., 2013, Quartuccio et al., 2013). In addition to BAFF, serum levels of APRIL were found increased in SS in relation to controls and both were positively correlated with focus score (Jonsson et al., 2005). Nocturne et al., (2015) reported elevated levels of BCA-1 in SS patients with NHL. The levels of several cytokines in SS patients with and without germinal centres were explored (Reksten et al., 2009, Szodoray et al., 2005).

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